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RE: Final Technical report for Award #N00014-05-1-0807

To whom it may concern:

Attached please find the final technical report for award #N00014-05-1-0807, *Non-Toxic Glycopeptide Analgesics for Combat Casualty Care*. The period of performance was June 13, 2005 through June 30, 2008.

Please let me know if there is any further information you require to close this grant out.

Sincerely,

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cc: Naval Research Laboratory, Defense Technical Information Center and ONR REG San Diego - N66018

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Background: Endogenous opiate peptides (enkephalins and endorphins) are more potent and specific than morphine and congeners. Specificity is determined by the "address segment" while binding is determined by the "message segment." Incorporation of carbohydrates into the address segment results in improved biodistribution and enhanced penetration of the blood-brain barrier (BBB). Thus, glycosylation of mu- or delta-selective peptides allows the resulting glycopeptides to be used as potent and safer alternatives to classical opiates such as morphine. Morphine and other muselective agonists are immunosuppressants, while the glycopeptide enkephalins are selective delta-agonists, which are known to be immunostimulants.

Hypothesis: Enkephalin transport and penetration of the blood-brain barrier can be determined by reversible binding to membranes, which may be manipulated by altering the amphipathicity of the address segment directly by introduction of hydrophilic sugar moieties, and lipophilic side chains.

Conclusion: Glycopeptide analogues of enkephalins are viable drug candidates. Obstacles regarding the synthesis and design of analgesics have been overcome. Further studies are required in order to meet FDA requirements. See attached .pdf file (14 pg and 22 pg reviews) for details.

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Delta-Selective Glycopeptides Related to Enkephalin Produce Profound Analgesia with Reduced Side Effects in Mice

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ANALGESICS BASED ON ENDOGENOUS NEUROPEPTIDES

The use of endogenous neuropeptides such as enkephalins and endorphins as drugs has remained an elusive goal since the 1970's. The principle reason for this is that peptides generally do not cross the blood-brain barrier, and are quickly degraded in the blood stream prior to delivery to opiate receptors in the brain. Animal research with glycosylated enkephalins and endorphins (dynorphins) indicates that potent analgesia is possible after intravenous or sub-cutaneous injection. Glycopeptides derived from delta-selective opioid agonists have 2-3X the potency of morphine, and lack many of the side effects associated with mu-agonists such as morphine. Morphine is still used on the battlefield for combat casualty care, and it is anticipated that further development of the glycopeptide analgesics will result in superior analgesics with greatly reduced side effects. Recent developments in this area are reported.

1.0 INTRODUCTION

There is no question that the American Civil War was a medical learning experience for the doctors involved in it. The American Civil War provided the setting for the first genuinely effective care of combat casualties with the introduction of field hospitals on or near the battlefield, and early treatment of casualties. (Figure 1) The pharmacopoeia of the day was not extensive by today's standards, but among the most effective agents were ether and chloroform anaesthetics used during amputations and other procedures, and morphine, used for the treatment of pre- and post-operative pain.[1] In the South, the scarcity and expense of imported drugs forced the Confederate Army to establish several medical laboratories to manufacture drugs for military use.[2] Empirical testing in military hospitals helped determine the clinical value of indigenous remedies. During this war morphine, both in its pure form and in various impure preparations of opium, gained its first widespread use on the battlefield, and in hospitals far removed from the field of battle. While there have been many advancements and refinements in combat casualty care in the intervening 130 years, morphine and its congeners are still used extensively, with many of the same unwanted side effects that were noted by the physicians of the 1860's. Chief among these unwanted side effects were respiratory depression and lowered blood pressure. It will never be known for certain, but it is very likely that opiates given to Stonewall Jackson in the course of his "diligent care" contributed to his death 8 days after the successful amputation of his left arm. It has recently been concluded that hemorrhagic shock and pneumonia, both possible sequellae of opiate administration, contributed to the death of this Confederate general, and consequently dealt a serious blow to the Confederate cause.[3] The problems of opiate induced respiratory depression are followed closely by the problems associated with tolerance and physical addiction. So widespread was the problem of opiate addiction of former soldiers after the war that it was given the term "veteran's disease."

Paper presented at the RTO HFM Symposium on "Combat Casualty Care in Ground Based Tactical Situations: Trauma Technology and Emergency Medical Procedures", held in St. Pete Beach, USA, 16-18 August 2004, and published in RTO-MP-HFM-109.

Figure 1: Mathew Brady recorded the medical treatment of Union casualties in the American Civil War. Amputations were performed in the field (left), either with or without the benefit of chloroform or ether as an anaesthetic. If the wounded were lucky enough to make it to a hospital (right), pain was generally treated with various preparations of opium or morphine. At the time of the Civil War, and afterwards, opiate addiction was referred to as "the veteran's disease."

2.0 ENDOGENOUS OPIOID PEPTIDES

Long before the discovery of the opioid peptides, it was suspected that mammals produced an endogenous substance with morphine-like effects. Eventually, with the aid of immunocytochemistry, these substances were discovered, and eventually isolated and chemically characterized. Three major classes exist: the relatively large dynorphins and endorphins (sometimes collectively referred to as endorphins), and the much smaller enkephalins (methionine enkephalin and leucine enkephalin). All of these peptides are enzymatic hydrolysis products of much larger precursor proteins that have a wide variety of neuropeptides embedded within their sequences. The enzymatic cleavage of these precursor peptides into the neurotransmitters and neuromodulators that are secreted by neurons allows for many pathways for regulation, and is a complex issue that will not be discussed here.[4]

3.0 ENHANCED STABILITY AND BBB TRANSPORT OF GLYCOPEPTIDES

With the discovery of the endogenous opiate peptides in the 1970's, and the recognition of their high selectivity and potency, it was initially anticipated that a new pharmacopoeia based on met-enkephalin, leu-enkephalin, or β-endorphin would emerge. Since these peptide opiates are degraded to pharmacologically inert amino acids, whereas morphine and similar alkaloidal pharmaceuticals produce a cascade of biologically active metabolites, it was logically (and correctly) assumed that peptide analgesics would possess a limited side effect profile. Problems associated with the physicochemical features of peptides, including their metabolic liability have been largely solved in the intervening years with the introduction of un-natural and/or D-amino acids, and by covalent modifications of the peptide backbone. Unfortunately, the pharmacodynamic behaviour of most peptides is still poor, and the blood-brain barrier (BBB) remains as a significant and largely unsolved deterrent to the effective delivery of peptide-based central analgesia. The BBB is not only a physical barrier represented by the tight junctions of the cells of the brain microcapillaries, but is also an enzymatic barrier caused by a broad spectrum of proteolytic enzymes and specific peptidases.

A significant advance was made in the transport of enkephalins was reported in 1994, when it was noted that glycosylated enkephalins penetrate the BBB to produce centrally mediated analgesia in mice after *i.v.* injection. A series of glycopeptides were synthesized[5] with varying types of O-linked glycosides attached to

Ser[6] of a potent δ-selective sequence first studied by Roques[6] (**Figure 2**). O-Linked glycosylation of the relatively lipophilic Leu-enkephalin C-terminal amide YdGFS*-$CONH_2$ led to enhanced surfactant properties[7] of the molecule, which in turn led to increased interaction with membranes and membrane mimics.[8] Although these relatively short glycosylated neuropeptides had no defined conformation in aqueous solution (*e.g.* they existed as random coils), in the presence of sodium dodecyl sulphate (SDS) micelles or other membrane mimics they adopted a very restricted and well-defined set of conformations, as indicated by circular dichroism (CD) and ^{1}H-NMR analysis.[9]

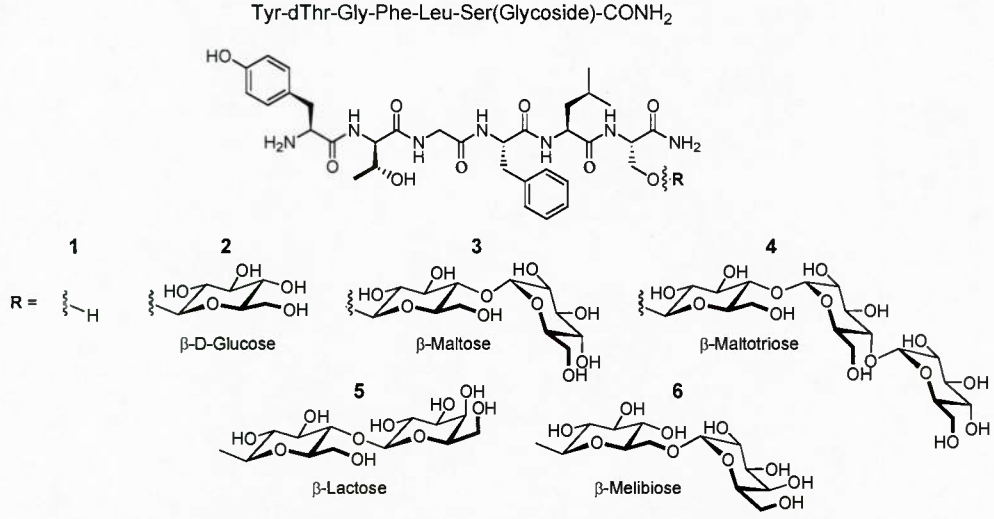

Figure 2: Glycosylated Enkephalin Analogues. Glycosyl hexapeptides were synthesized using solid-phase Fmoc chemistry. The Fmoc serine glycosides were incorporated as the peracetates, and synthesized using methods developed in the Polt group.

YdGFLS*-$CONH_2$ Glycoside	Glucoside Moiety	δ Binding (nM)	μ Binding (nM)	MVD IC_{50} (nM)	GPI IC_{50} (nM)
1 (peptide control)	—	2.1	7.5	2.7	25
2 (glucomorphin)	β-D-Glc	2.4	7.6	1.6	34
3 (maltomorphin)	α-D-Glc-(1—>4)-β-D-Glc	9.9	30.8	1.7	52.6
4 (maltotrimorphin)	[α-D-Glc-(1—>4)]$_2$-β-D-Glc	3.8	15	7.7	71.7
5 (lactomorphin)	β-D-Gal-(1—>4)-β-D-Glc	17.3	40	5.72	34.8
6 (biomorphin)	α-D-Gal-(1—>6)-β-D-Glc	5.6	36.6	6.06	43.8

Table 1: *In Vitro* Binding Activity and Functional Assays for Glycosylated DTLES. IC_{50}'s for δ- and μ-opioid binding were determined using displacement of ^{3}H-labeled radioligands from rat brain homogenates. Functional assays were performed using electrically stimulated mouse *vas deferens* and *guinea pig illium.*

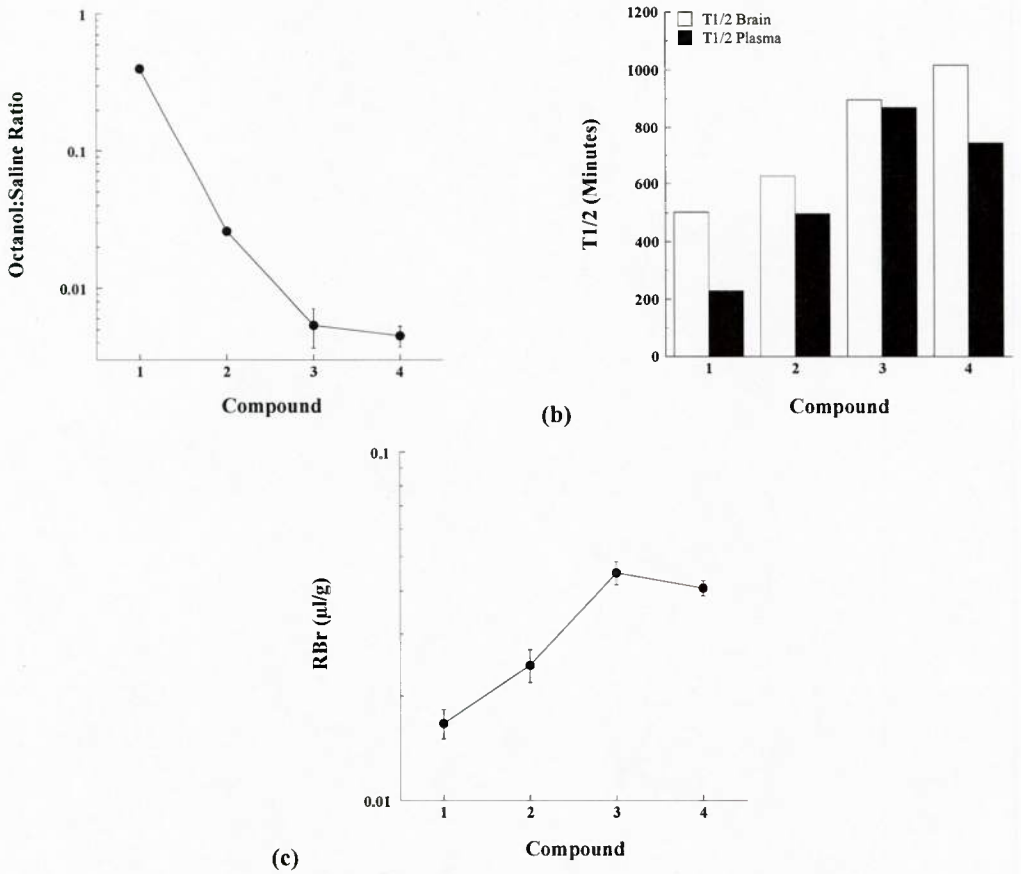

Figure 3: Glycopeptide Stability and Transport. (a) Octanol-saline distribution for the unglycosylated peptide 1 and glycopeptides 2, 3, and 4. The addition of 1, 2, or 3 glucose units to the opioid peptide message significantly decreases lipophilicity. (b) The *in vitro* stabilities of the peptide and glycopeptides were measured in mouse brain and serum. Increased glycosylation led to significant increases in stability in both brain and serum. Brain stability increased with each additional glucose. However, in the serum, the stability of the trisaccharide was lower than that of the disaccharide. (c) Brain delivery of the peptides measured by *in situ* perfusion studies. Addition of glucose to the peptide significantly increased uptake. Uptake to the brain was improved further for the disaccharide, giving the maximal delivery. The trisaccharide produced no further increase in BBB penetration.

Classical pharmacological theories of BBB transport suggest that peptides are not lipophilic enough to diffuse into the brain.[10] Glycosylation decreases lipophilicity even further. Despite this, greatly increased transport rates in rat brain have been observed for the glycosylated enkephalins (**Figure 3**). Previous studies with the glucoside **2** indicated that the increased transport was due to a saturable mechanism, thus further ruling out simple diffusion. Reversible interaction of the glycopeptides with the membrane is believed to promote transport through the brain capillaries by transcytosis.[11] Several other possible modes of transport (simple diffusion and receptor-mediated processes) have been ruled out.[12] Maximum transport rates (and maximum biological effects) are observed when the optimum degree of glycosylation is achieved. For this peptide, the disaccharide produces both the optimal transport and stability *in vivo*. In general, glycosylation leads to

enhanced stability of the peptide "message" in both serum and brain. The identity of the individual sugars does, however, contribute to the overall biological effect, which is a product of both BBB transport rates and the stability of the peptide in serum, as well as metabolism and excretion by the liver and kidneys.

4.0 ANALGESIC EFFECTS OF GLYCOSYLATED ENKEPHALINS

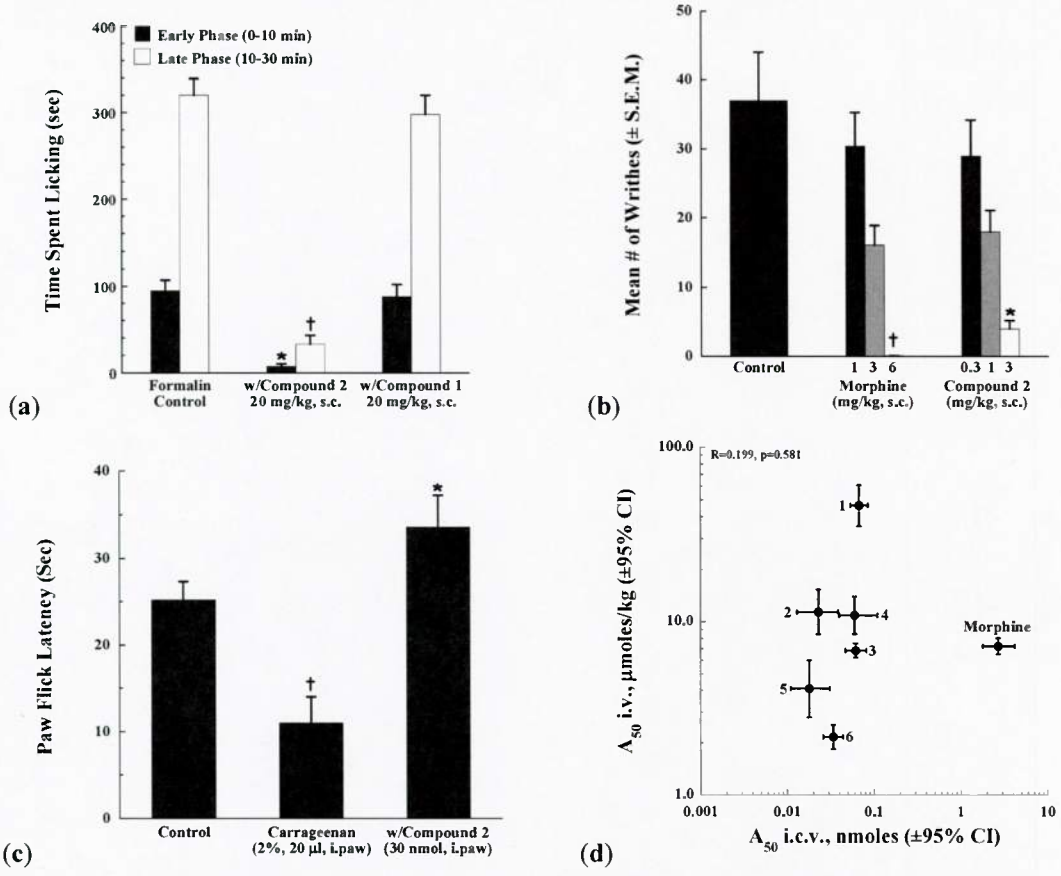

Figure 4: Antinociception *in vivo*. The glycosylated enkephalins showed strong analgesic activity in tests of antinociception after peripheral administration, which are more clinically relevant than the tail flick assay. (a) Mouse formalin paw test, glycopeptide 2, *s.c.* (b) Mouse abdominal constriction test, glycopeptide 2, *s.c.* (c) Mouse paw inflammation test with carrageenan, glycopeptide 2, *i. paw.* Injection of glycopeptide 2 into the contralateral injection had no antinociceptive effects. (d) Antinociceptive effects (mouse tail flick) of glycosylated enkephalins (A_{50} values) after *i.c.v.* injection (X axis), and after *i.v.* injection (Y axis). Morphine has been included as a reference point, but has been excluded from the correlation values, shown on the upper left part of the diagram. The observed analgesia after *i.v.* injection correlates most strongly with glycopeptide stability (Fig. 3b), and brain transport values (Fig 3c), rather than the *i.c.v.* potency.

The extent of antinociception was shown to be comparable to, or even superior to the effects of morphine in mice after *i.c.v.* and *i.v.* administration[13] using the warm water tail flick assay.[14] The representative glycopeptides all produced full agonist effects in these assays with the potencies exceeding that of morphine

on a μmol/kg basis in some cases. (**Figure 4**) Additional analgesic assays involving visceral, chemical and inflammatory pain states were also used to gauge the effectiveness of **2** and **5** after *i.v.* and *s.c.* injection.

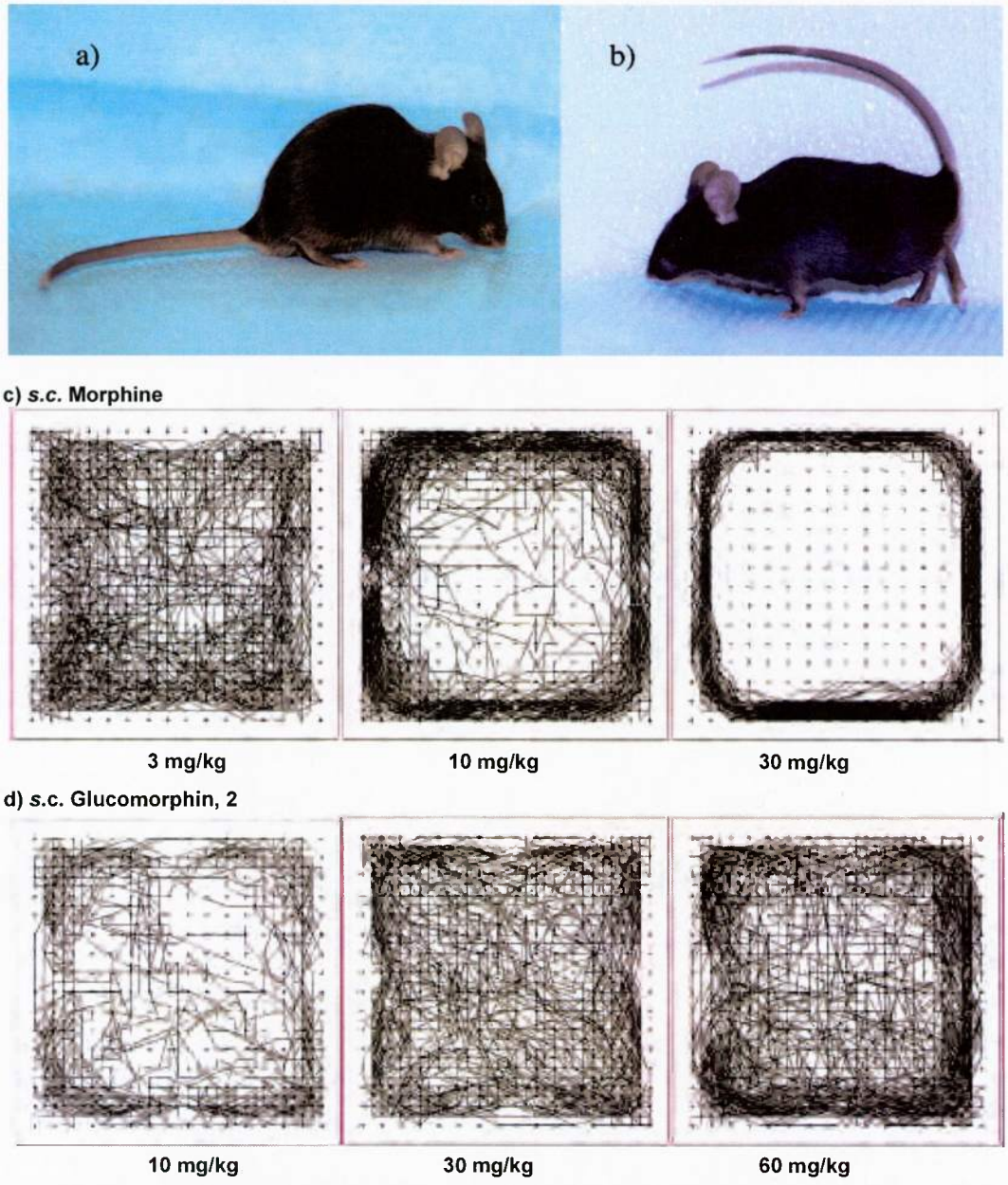

Figure 5: Non-analgesic effects of opioids on mice. Both mice have received equi-analgesic (A_{90}) doses of drug. (a) and (b) Glycopeptide-based analgesia did not induce Straub tail. (c) Morphine-induced analgesia induced large increases in locomotor activity, stereotypic circling, compared to equi-analgesic doses of glycopeptide 2 (d).

Two well-known effects of morphine in rodents are increases in locomotor activity[15,16] with stereotypic patterns of movement,[17] and increases in muscular rigidity, including Straub tail.[18] Unlike morphine and other μ-selective opioids, at equivalent *s.c.* A_{90} antinociceptive doses, or even supramaximal doses, the glycopeptide analgesics produced minimal increases in locomotor activity, and did not produce Straub tail (**Figure 5**). These results were confirmed in two different strains of out-bred mice.

5.0 MECHANISM OF TRANSPORT

Evidence obtained from *in vivo*[19,20] as well as *in vitro* experiments[9] with the glycopeptides are consistent with an endocytotic mechanism of transport. Receptor mediated transport and diffusive mechanisms have been ruled out, and further work strongly suggests that adsorption to the endothelial membrane of the brain capillaries is required for BBB transport. While the drug must adsorb strongly to the membrane in order to undergo endocytosis or transcytosis, this must also be a reversible adsorption, otherwise the drug will bind tightly to the first membrane it sees, resulting in poorer transport. This concept is demonstrated clearly with the amphipathic ∝-helices, **14**, **15**, and **16**. (Table 3)

Our work began with glycosylated enkephalins that were designed to have potent δ-agonist activity, but still have appreciable μ-agonist activity. While it is possible to produce some analgesic effects through the δ-receptor alone, previous work has shown that μ-agonists are much more effective in this regard. It was hoped that mixed δ/μ-agonists would show reduced side effects, relative to μ-selective agonists, *e.g.* morphine. Other researchers have proposed μ-agonist/δ-antagonists as drug candidates for analgesia with reduced side-effects.[21] An important aspect that is not fully understood is the role that "address" segments play in determining receptor selectivity.

Helices are the most commonly occurring secondary structural elements in globular proteins, accounting for one-third of all the residues.[22] Linus Pauling first proposed the α-helix as an important motif of secondary structure in proteins in 1948,[23] interestingly, without any experimental evidence.[24,25] Segrest first theorized the *amphipathic* (a.k.a. amphiphilic) helix to be an important structural motif of integral membrane proteins in 1974.[26] It is estimated that over 50% of all α-helices in nature are amphipathic.[27] These proteins are unique in that they possess hydrophobic and hydrophilic parts either by primary structure (highly hydrophilic N-terminus and hydrophobic C-terminus) or by secondary structure, with polar residues pointing one to face and the non-polar residues on the opposite face. This allows them to "float" in a cell membrane, exposing the hydrophilic side to the aqueous exterior of the cell and the hydrophobic side to the lipophilic membrane.[28,29] This peptide-membrane interaction is believed to be important for two reasons. First, the amphipathic nature of the helix can help guide a drug or hormone to its specific receptor by narrowing the receptor search from a 3-dimensional search to one in 2-dimensions. Surface-assisted "reduction-of-dimensionality" calculations, performed by Polya in 1921, were examined by Max Delbrück in which he quantitatively demonstrated the viability of this theory.[30] Assuming that no other forces are at work (*e.g.* convection), and that the membrane is fluid, the probability of a substrate finding its corresponding receptor is much better in 2-dimensions (*e.g.* a cell surface) than in 3 (*e.g.* in solution)— almost 100% when the search is reduced to 2-dimensions.

Second, membrane insertion may allow the portion that interacts with the receptor (pharmacophore or "message") to be fixed in a specific geometry. By restricting mobility in the membrane near the binding site, the amphipathic α-helix can dramatically alter the peptide-receptor interaction.[31] In addition, membrane insertion can also induce a specific conformation in the ligand, different from its solution conformation. It seems clear that the bioactive conformation of a peptide is the membrane-bound conformation, and that membrane insertion is actually the first step in receptor activation.

The endogenous neuropeptide β-endorphin is a 31-residue naturally occurring opioid peptide. The first 5 residues of β-endorphin are identical to Met-Enkephalin. It has been shown that the α-helical structure of C-terminal region of β-endorphin plays a role in the receptor binding & opiate activities, and resistance to proteolysis.[32] Kaiser[33] proposed that β-endorphin consists of the [Met^5]-enkephalin peptide sequence at the N-terminus, a hydrophilic linker region from residues 6—12, and an amphiphilic helical region between the residues Pro^{13} and Gly^{30}, which were assumed to be "helix breakers." This hypothesis has been supported by the conformational analysis of a number of β-endorphin mimics with artificial C-terminal helical regions with amphipathic character.[34,35,36] All of the analogues were α-helical by CD measurements, as the monomer or oligomers, and showed strong opioid agonism *in vitro* when compared to natural β-endorphin. These studies clearly suggest that amphipathicity of the entire peptide is more important than the identity of specific amino acids present in the helical C-terminus.[37] This has been further supported by the work of Kyle,[38] who synthesized several potent peptide analogues containing the α-helix-promoting residues α-aminoisobutyric acid (Aib) and N-methyl alanine (MeAla) near the C-terminal region of nociceptin, the natural ligand for the recently identified opioid receptor-like 1 receptor (ORL-1). According to Schwyzer,[39] the N-terminal "message" is steered toward certain receptors and away from others by the C-terminal "address" segment, which interacts with the membrane to orient the message with respect to the receptor.

Dynorphin A (1-17) is an endogenous opioid heptadecapeptide which binds preferentially to the κ opioid receptor.[40] Dynorphin consists of a N-terminal message identical to Leu-enkephalin, and an address sequence that imparts selectivity for κ receptors.[41] Dynorphin A is believed to adopt an extended and/or random coil structure as determined by various spectroscopic measurements.[42,43,44,45,46] In the presence of DPC micelles Dynorphin A is believed to contain a less ordered N-terminus, a well defined α-helix segment spanning between Phe^4 and Pro^{10} or Lys^{11}and a β-turn from Trp^{14} to Gln^{17}.[47] Based on NMR results, the authors concluded that both the α-helix and the C-terminal β-turn may be a consequence of dynorphin's interaction with the micelle, and may be important structural features of the full-length peptide when bound to the cell membrane in vivo. The α-helix could have multiple roles in positioning the amphipathic helix for interaction with the receptor, as amphipathic helices have many roles at interface.

Helix Glucoside	Glycopeptide Sequence	Retention Time (RP-HPLC)	% Helicity (CD)	*i.c.v.* Analgesia IC_{50} (*pico*Mol)
7	YtGFLGELAS*KWFNALE	8.85 min	69%	insoluble
8	YtGFLGELAS*KWFNALES*	7.95 "	55%	270
9	YtGFLGELAS*KWFNALES*F	9.91 "	53%	insoluble
10	YtGFLGELAS*KWFNALES*FW	12.48 "	68%	insoluble
11	YtGFLGLLKS*FAES*WS*NF	6.69 "	34%	~ 30
12	YtGFLGKS*FAELWS*NFLS*	5.35 "	14%	~30
13	YtGFLGLLKS*FWES*WS*NF	8.25 "	37%	~30

Table 2: Glycosylated Endorphin Analogues.

Helix Glucoside	Glycopeptide Sequence (3rd Generation)	Retention Time (RP-HPLC)	Per Cent Helicity (CD)	MVD IC_{50} (nM)	GPI IC_{50} (nM)
14	YtGFL(**P**)NLBEKALKS*L-$CONH_2$	31.57	21	34.5	63.1
15	YtGFL(**βA**)NLBEKALKS*L-$CONH_2$	33.50	26	23.0	354
16	YtGFL(**GG**)NLBEKALKS*L-$CONH_2$	30.30	14	18.8	196
—	Morphine	—	—	258	54.7

Table 3: Glycosylated Endorphin Analogues.

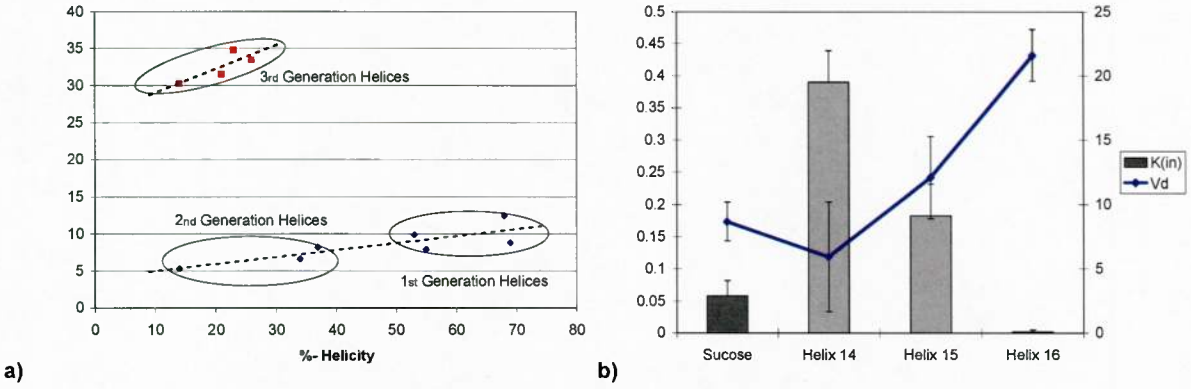

Figure 6: (a) Plot of Retention Time *vs* Degree of Helicity. (b) Mouse BBB Transport Data.

The first- and second-generation endorphins were also based on the δ-selective YdGFL~ opioid message. Formed by simple truncation, the first generation helices, 7—**10**, were designed to probe the minimum length for helix formation. Essentially, we overshot the target, and all of these compounds were extremely helical, but they were not water soluble enough to work with, with the exception of helix **8**. This compound possessed appreciable antinociceptive activity, however.[48] All of these compounds were quite soluble in the presence of SDS micelles. Since these compounds are so stable in their helical form, they probably form aggregates, and fall out of solution in the absence of the detergent. The second generation helices, **11**—**13**, were designed to be less lipophilic, and consequently were more water soluble, and showed much less helicity in the presence of micelles.[49]

The third-generation helical endorphin-based glycopeptides, **14**—**16**, used the same δ-selective peptide DTLET first studied by Roques, and showed much superior properties, both in the chemistry lab and in the mouse. Using *in situ* methods in the mouse, not rat studies as before, Egleton was able to measure BBB transport rates independently of analgesia, and Bilsky has been able to demonstrate the analgesic effects of these larger glycopeptides using *i.c.v.* tail flick results in the mouse.[50] Initial studies with these glyco-hexadecapeptides indicated that BBB transport rates were determined by the amphipathic nature of the glycopeptides,[51] rather than the lipophilicity of the compound, *per se*,[52] and that they actually show BBB transport rates that are similar to, or better than the shorter enkephalin analogues.

These endorphin analogues have the same N-terminal YdGFL~ opioid message contained in the enkephalin analogues **1**—**6**, and the same C-terminal amide address sequence ~NLBEKALKS*L-$CONH_2$, where B is the helix-stablizing α-aminoisobutyric acid (Aib) residue, and S* is the serine glucoside residue. The "linker region," which is intended to "break" the helix, and prevent propagation of the helical address into the opioid message, is different in the three glycopeptides: **14** => proline, **15** => β-alanine, and **16** => glycylglycine.

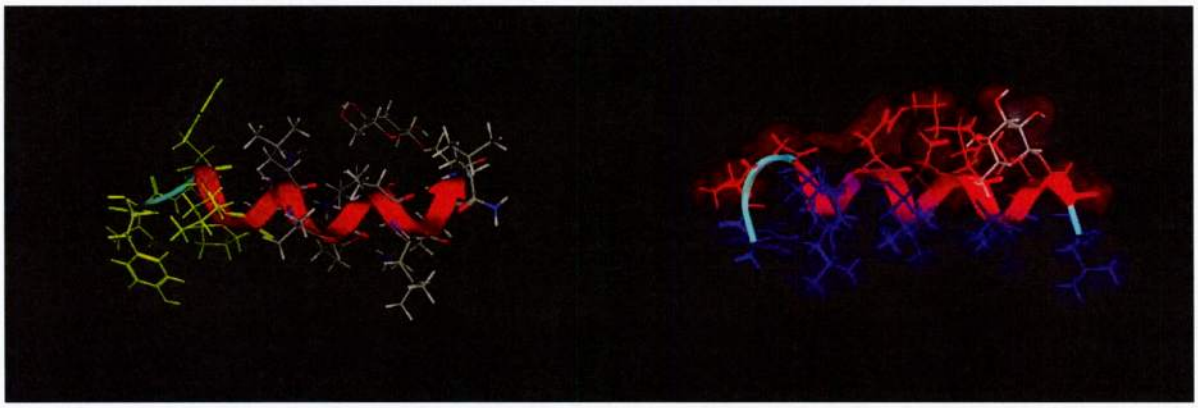

Figure 7: Lipid Bound Helix. One structure of glycopeptide 14 in the presence of micelles, as determined by NOE-constrained molecular dynamics calculations. The message segment is labelled in yellow, and the helix indicated with the overlaid ribbon. The structure on the right has the hydrophobic (blue) and hydrophilic (red) surfaces labelled. The structures were rendered with the MOE® software package.

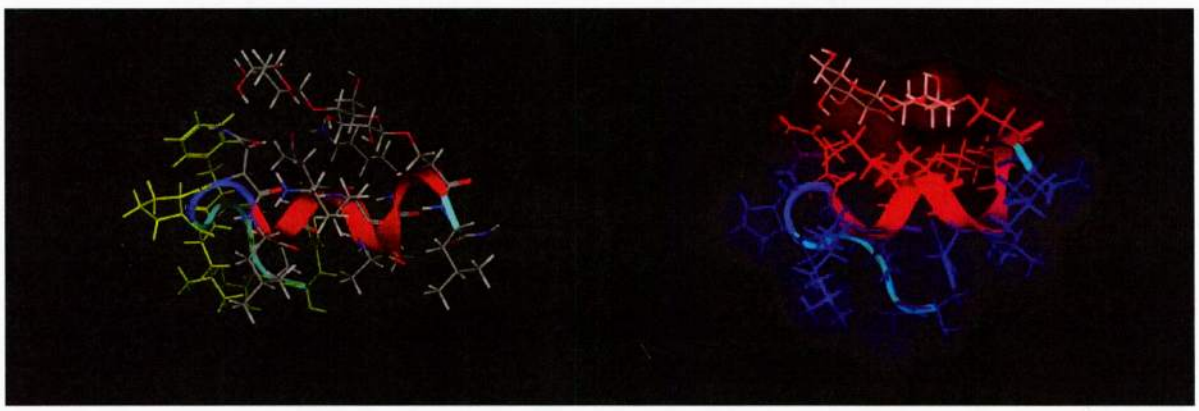

Figure 8: Lipid Bound Helix-Bend. One structure of glycopeptide 17 in the presence of micelles, as determined by NOE-constrained molecular dynamics calculations. The message segment is labelled in yellow, and the helix indicated with the overlaid ribbon. The structure on the right has the hydrophobic (blue) and hydrophilic (red) surfaces labelled. The structures were rendered with the MOE® software package.

While the data presented in Figure 6 is interesting, and perhaps even compelling, it is also clear that one cannot only use the degree of helicity to predict amphipathicity. NMR evidence, in conjunction with Monte Carlo calculations (NOE constraints not discussed here) shows that the glycopeptides bind to micelles, and

adopt a very restricted set of conformations. For the helices **14**, **15**, **16**, and the disaccharide **17** (not pictured in Table 3, but is the top-most data point in Figure 6a) we see two membrane bound conformational ensembles, one that is very helical, (*e.g.* Figure 7) and one that has a helix-bend motif (*e.g.* Figure 8), but is none-the-less very amphipathic. The peptide sequence for **17** is the same as the sequence for **14**, but the compounds differ in that **14** is glycosylated with the β-D-glucoside, and **17** is glycosylated with the disaccharide β-lactose. These two compound both show the same conformations in their micelle-bound ensembles based on NMR, and similar helicities based on CD, but slightly different population densities.

While it there is still much to be learned about the details of both the transport and binding processes of the amphipathic glycopeptides, an important principle has emerged concerning transport. It seems clear that one must have a glycopeptide that essentially has two states: 1) A state defined by one or more membrane-bound conformations that permit or promote endocytosis. 2) A state defined by a water-soluble, or random coil state that permits "membrane hopping." The key to efficient transport is to balance these two states so that the compound is neither retained in the membrane, or held in solution so that it cannot undergo adsorptive endocytosis. It may also be true that aggregation of glycopeptides on a membrane surface may actually initiate and promote endocytosis.

6.0 CONCLUSIONS

Based on the results obtained so far, it would seem that further pre-clinical studies are warranted to test the viability of the glycosyl enkephalin analogues (*e.g.* compounds **2**, **5** or **6**) as a replacement for morphine on the battlefield. Anecdotal studies in mice suggest that these compounds possess an extremely low level of toxicity, even at super-analgesic doses. The notion that one could administer a large sub-cutaneous dose of a non-toxic glycopeptide that would have prolonged analgesic effects without respiratory depression or the risk of overdose is particularly appealing. Further research needs to be completed in order to quantify the effects of the glycosylated δ-agonists on respiration and blood pressure, particularly in hypovolemic animals to gauge the propensity of these compounds to induce hemorrhagic shock. Complete absorption, metabolism and excretion studies (ADME) need to be completed, and oral bioavailability needs to be explored. The fact that the glycosylation strategy seems to be effective with the much larger endorphin analogues (*e.g.* compounds **14** and **15**) suggest that this approach may have general applicability to BBB transport of non-analgesic (or even non-opioid) neuropeptides, which could lead to novel treatments for anxiety, stress-related disorders and depression.

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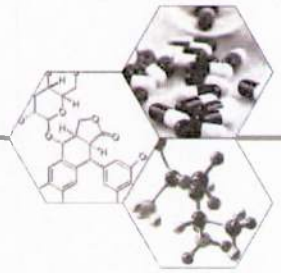

Opioid glycopeptide analgesics derived from endogenous enkephalins and endorphins

Over the past two decades, potent and selective analgesics have been developed from endogenous opioid peptides. Glycosylation provides an important means of modulating interaction with biological membranes, which greatly affects the pharmacodynamics and pharmacokinetics of the resulting glycopeptide analogues. Furthermore, manipulation of the membrane affinity allows penetration of cellular barriers that block efficient drug distribution, including the blood–brain barrier. Extremely potent and selective opiate agonists have been developed from endogenous peptides, some of which show great promise as drug candidates.

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The success rate of CNS drug development is lower than that of other therapeutic areas [1]. There are multiple reasons for this dearth of new drugs, including the sheer complexity of the brain and its neuropathologies; a propensity for CNS drugs to cause CNS-mediated side effects that limit dosing and compliance; a lack of validated biomarkers to inform whether a given neutothetapeutic agent is engaging the target in sufficient concentrations to modulate the CNS target; and the presence of the blood–brain barrier (BBB), across which CNS agents need to penetrate. Among these challenges, the BBB is considered to be most problematic for peptide or protein-based therapies [2–4]. However, peptides and proteins do offer distinct advantages for developing efficacious and well-tolerated treatments for CNS diseases, such as chronic pain, Alzheimer's disease and Parkinson's disease. These advantages include intrinsic affinity and selectivity of the peptide for native receptors and the metabolism of the peptide into smaller fragments and amino acids (versus the variety of active metabolites seen with small molecules). In addition, peptides and proteins can bind to multiple sites across a receptor protein, offering greater opportunity to fine tune the receptor–effector response [5,6]. Historical challenges for peptide and ptotein drug development are being addressed, including modifications that increase stability, techniques that increase yields and lower total synthetic costs, and technology to improve tissue targeting, including access to the CNS. A number of monographs have been written since the beginning of this century that describe formulations and articulate solutions of increasing sophistication to address the problems of peptide-based drugs [7–9].

Opioid receptors

The classical opioid receptors [10] are divided into three subtypes, the μ receptor (MOR or MOP), the δ receptor (DOR or DOP) and the κ receptor (KOR or KOP). Another receptor, the nociceptin or orphanin receptor (NOP or ORL1), is widely distributed in the CNS, and is clearly related to the opioid receptors in terms of its molecular biology but is generally not regarded as an opioid receptot as it does not respond to classical opioid agonists or antagonists. IUPHAR recommends use of the terminology MOP, DOP, KOP and NOP, replacing the older recommendation for OP_1 and OP_2, fot example [201]. All of the opioid receptors have been cloned from various species, including mouse, rat and human. Opioid receptors are GPCRs that consist of highly homologous seven-transmembrane helical domains, and are linked with extracellular peptide loops of very limited size (**FIGURE 1**) [11,12].

The MOP receptor [13] is distributed presynaptically in various brain regions, including the limbic structures, the brainstem (i.e., the periaqueductal grey area) and in the superficial dorsal horn of the spinal cord. It was initially chatacterized in functional smooth muscle preparations of the guinea pig ileum (GPI). This receptor remains the principal target of opioid analgesics currently in clinical use, with the μ referring to morphine. The MOP receptor is widely distributed in other areas of the brain, as well as non-CNS tissues, most relevantly in the immune system, and the cardiovascular and gastrointestinal systems. Pharmacological activation of MOP not only modifies the transmission and perception of nociceptive stimuli, but also a host of other effects, including reduced respiratory drive in response to increased levels

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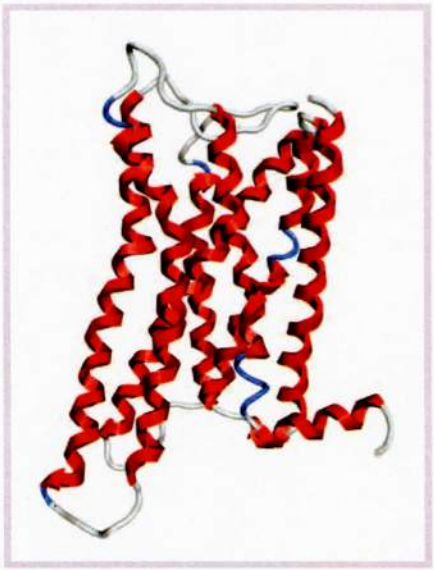


Figure 1. Opioid receptors. The μ-opioid receptor G-protein-coupled receptor derived from bovine rhodopsin by homology modeling [12].

of CO_2, opioid-induced bowel dysfunction, abuse liability and pruritus (itching). Repeated or prolonged MOP activation results in adaptations that manifest as tolerance and physical dependence, further complicating management of chronic pain sufferers and patients with substance abuse disorders. Mixed opioid agonists/antagonists and partial MOP agonists have been used to limit some of the opioid side effects with a mixed degree of success. Clearly, opioid pharmacology is a complex issue [14].

The DOP receptor [15] was originally characterized using the mouse vas deferens smooth muscle tissue preparation (mouse *vas deferens* [MVD] assay) [16]. The receptor is widely distributed anatomically, being found in the same general anatomical areas as MOP. DOP also contributes to a variety of physical and emotional effects, including initiation of movement [17], regulation of pain and reward circuitry, as well as other complex CNS behaviors (mood/affect and anxiety). The issues of cellular colocalization of DOP and MOP, as well as the formation of functional 'heterodimers' continue to be the subject of considerable interest [18–20]. Despite the progress that has been made in the localization of opioid receptors [21], the precise localization and neuronal and cellular pathways through which these three receptor types work remains incompletely understood [22,23]. Although DOP receptors contribute to analgesia, euphoria and physical dependence, DOP agonists may be able to produce broad spectrum analgesic efficacy with reduced propensity to produce classic opioid side effects [24].

The KOP receptor, named after the κ-agonist ketocyclazocine, is not as well studied as MOP and DOP, although it has attracted interest from pharmaceutical companies, as either a standalone drug [25,26] or in conjunction with agonism of other opioid receptors [27]. Stimulation of CNS KOP receptors is generally associated with dysphoria and psychomimetic effects, along with limited analgesic efficacy, especially in men [28]. Peripherally active KOP agonists produce antinociception in animal models of pain [29] and are being explored as targets for analgesia in several chronic pain states [18,30,31]. KOP antagonists are also being investigated as treatments for addiction and depression [32].

The diversity of the endogenous neuropeptides and their receptors provide many opportunities for drug discovery [33]. If there were a straightforward methodology for converting endogenous neuropeptides into useful CNS drugs, then a new and potentially sea-changing pharmacopeia would be available for the treatment of CNS disorders.

Endogenous opioid peptide agonists

Since the discovery of the two endogenous pentapeptides, Met-enkephalin and Leu-enkephalin in the 1970s, perhaps as many as 300 endogenous neuropeptides have been identified in widespread locations throughout the CNS **Table 1**. Endogenous opioid peptides and their receptors undergo modulation in response to various physiological conditions, such as inflammation, tissue injury, pain and other stressors [34].

The three classes of endogenous opioid peptides (enkephalins, endorphins or dynorphins) are typically assigned to the three types of opioid receptors (DOP, MOP and KOP, respectively). This approach is misleading since the absolute selectivity of each peptide is limited, and it neglects the fact that there are many cleavage variants of the neuropeptides, splice variants for the receptors and, likely, variations in their glycoforms. The endogenous peptides are not orthogonal and neuropeptide receptors might, for example, just as easily be thought of as 'metorphamide receptors' or 'enkephalin receptors' [35]. Moreover, α-endorphin and γ-endorphin have been found to be inactive at

Table 1. Endogenous opioid peptides[1].

Peptide	Sequence	Subtype
Enkephalins		
Leu-enkephalin	**YGGF**L	δ receptor/μ receptor
Met-enkephalin	**YGGF**M	μ receptor/δ receptor
Metorphamide	**YGGF**MRRV-NH_2	δ receptor/μ receptor
Peptide E	**YGGF**MRRVGRPEWWMDYQKR**YGGF**L_{25}	μ receptor/κ receptor
Endorphins		
β-endorphin	**YGGF**MTSEKSQTPLVTLFKNAIIKNAYKKGE$_{31}$	μ receptor/δ receptor
γ-endorphin	**YGGF**MTSEKSQTPLVTL_{17}	μ receptor/unknown
α-endorphin	**YGGF**MTSEKSQTPLVT_{16}	μ receptor/unknown
Dynorphins		
Dynorphin A	**YGGF**LRRIRPKLKWDNQ_{17}	κ receptor (μ receptor)
Dynorphin B	**YGGF**LRRQFKVVT_{13}	κ receptor (μ receptor, δ receptor)
$Dynorphin_{1-8}$	**YGGF**LRRQ_8	κ receptor (μ receptor, δ receptor)
α-neoendorphin	**YGGF**LRKYPK	κ receptor (μ receptor, δ receptor)
β-neoendorphin	**YGGF**LRKYP	κ receptor (μ receptor, δ receptor)
Nociceptin/orphanin FQ		
Nociceptin	FGGFTGARKSARKLANQ	ORL1
Endomorphins		
Endomorphin-1	YPWF-NH_2	μ receptor
Endomorphin-2	YPFF-NH_2	μ receptor
Dermal peptides		
Dermorphin	YaFGYPS-NH_2	μ receptor
Deltorphin A	YmFHLMD	δ receptor
Deltorphin C	YaFDVVG-NH_2	δ receptor

[1]A few of the opioid peptides isolated from the mammalian CNS and from the skin of amphibians are illustrated. Their common names, sequences and suggested receptor binding activities are shown. Weaker binding activities are in parentheses.

sites that are sensitive to β-endorphin [36] and other sites have been found that are sensitive to γ-endorphin, which have been referred to as 'non-opioid' in nature [37,38].

Schwyzer's membrane compartment concepts

Schwyzer articulated critical roles for the membrane in peptide–receptor binding events (**Figure 2**) [39]. Although his 'membrane compartment theory' may have overstated the influence of the membrane in differentiating receptor selectivity (μ vs δ vs κ), it is clear that the membrane environment does play critical roles in pre-organizing the peptide conformation prior to binding, as well as the peptide–receptor binding event itself [40]. We view binding as a three-step process:

- Adsorption of the peptide ligand to the membrane. This promotes receptor binding by reducing a 3D search for the receptor to a faster 2D search. Surface-assisted 'reduction-of-dimensionality' calculations, performed by Polya in 1921, were examined by Max Delbrück in which he quantitatively demonstrated the viability of this theory [41,42];
- Conformational changes in the peptide induced by the asymmetric environment of the membrane. Amphipathicity of the peptide is believed to reorganize the peptide–membrane aggregates into minimal energy states [43,44];
- The binding event itself. GPCR binding and activation is a complex, multifaceted phenomenon. It is, however, beyond the scope of this review.

The neurovascular unit & the BBB

In order to enter the brain and CNS, pharmaceutical agents must first penetrate the BBB (**Figure 3**). There are several strategies available to make a peptide metabolically stable but the transport of a peptide across BBB is still remains a major hurdle in developing CNS drugs [45]. Contrary to the belief that small molecules with molecular weights under 400 readily cross the

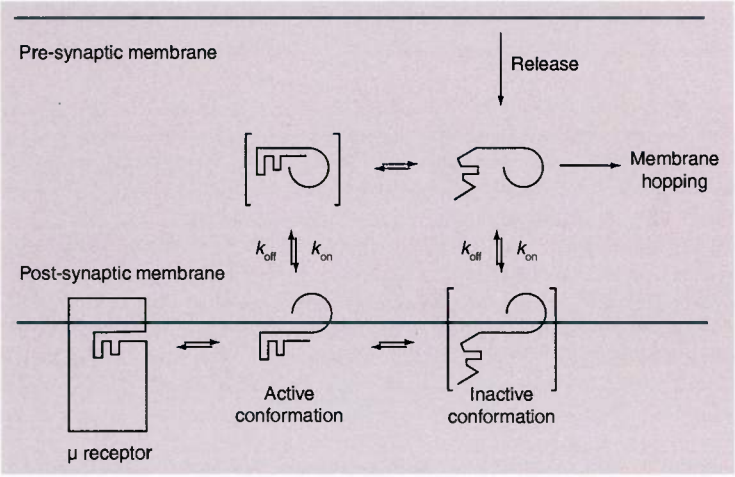


Figure 2. Membrane hopping. Endogenous opioids associate with the membranes (k_{on} >> k_{off}) and bind to one or more of the opioid receptors via a membrane-bound conformation (Fisher's lock and key). Studies show that active (folded) conformations are favored in the membrane and inactive (random coil) conformations are favored in the absence of a membrane. Incorporation of glycosides, represented by the 270° arc, can shift the k_{on}/k_{off} equilibrium to facilitate 'membrane hopping'.

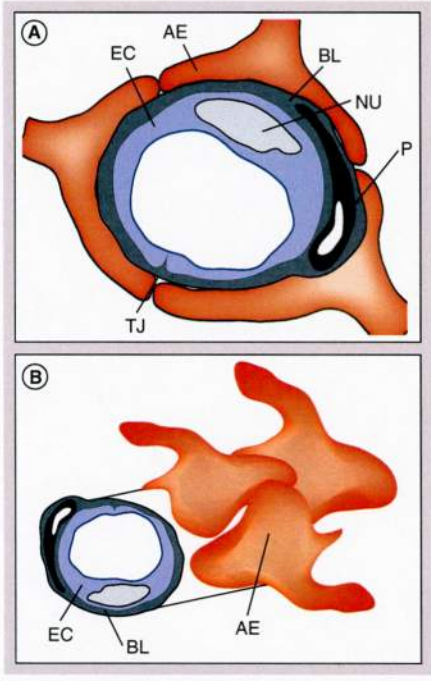


Figure 3. The neurovascular unit. The neurovascular unit forms the blood–brain barrier that prevents the passage of most peptides and other polar substances from the capillaries into the brain.

BBB, it has been observed that almost 98% of small molecules do not readily cross the BBB. Further, evidence has accumulated that peptides can penetrate the CNS by several different mechanisms [46–49]. These observations clearly indicate molecule size is not the primary factor, but rather the overall physiochemical characteristics of the molecule is critical for BBB transport. Since the BBB is composed of endothelial membranes, many research groups focus on designing peptides with increased lipid solubility. Simply increasing the lipid solubility of a drug molecule may have undesirable effects, such as decreasing solubility and bioavailability, and increasing plasma protein binding.

The BBB is composed of endothelial membranes that function as a continuous lipid barrier that protects the brain from toxic substances by preventing their entrance from bloodside to CNS. The BBB is also an enzymatic barrier that poses additional challenges in developing peptide/protein-based CNS drugs. Further, it has been viewed recently as a regulatory interface between the CNS and circulation with nutritional, homeostatic and communication functions. Understanding of the principles and physiology of BBB has improved a great deal in the past decade. Moreover, evidence is accumulating that many peptides and proteins cross the BBB in amounts sufficient to affect CNS function. It is now clear that the BBB is not an absolute physical barrier but a regulatory tool that controls the delivery of the substances to the CNS. Strategies based on this principle are proving to be very successful [50]. Other strategies using 'molecular umbrellas' [51], 'Trojan horses' [52] and BBB 'shuttles' [53] have been proposed by various groups. We have successfully applied glycosylation as a strategy to improve the BBB penetration, as well as the stability and systemic availability of enkephalins and the larger endorphin-like peptides.

Glycopeptide synthesis

Initially, *O*-linked glycopeptides were considered to be exotic substances, and were difficult to evaluate as drugs simply because the synthetic methods required to produce them in tangible amounts were lacking. In the last 40 years this situation has changed, however, glycopeptides are still significantly more difficult to produce than simple peptides, even if the requisite Fmoc–amino acid glycosides are commercially available, and particularly so if the glycosides require synthesis [54,55]. Enzymatic approaches

have been incorporated into chemical methods for serine and threonine glycosides [56]. The ideal methodology should produce high yields of pure diastereomers, typically β-anomers for the highest stability. Many classical methods produce high yields of the desired anomers, but require the production of labile glycosyl donors or very reactive (e.g., unstable) promoters [57].

Production of *O*-linked glucosides and lactosides of enkephalins and endorphins requires the corresponding acetate-protected glycosides of Fmoc–serine or Fmoc–threonine. The benzophenone Schiff base appeared to be a very good protection for amino groups of serine and threonine for glycoside formation [58]. *O*-glycosides of these amino acids with different monosaccharides, aminosugars and deoxysugars were obtained with excellent yield and very high stereoselectivity [59]. Glycoside peracetates have been used as building blocks for solid-phase glycopeptide synthesis, and significant improvements have been made in the production of the glycoside building blocks [60]. An even more efficient and direct approach to these precursors has now been developed that proceeds directly from Fmoc–serine or Fmoc–threonine as glycosyl acceptors, and either β-D-glucose peracetate or β-lactose peracetate in the presence of 'minimally competent' Lewis acids, such as indium(III)bromide **(FIGURE 4)** [61].

The glycopeptides can be synthesized manually based on established solid-phase *N*-fluorenylmethoxycarbonyl methods (Fmoc chemistry) **(FIGURE 5)**. The side chain-protected amino acids used by our research group were Fmoc–Lys(Boc)-OH, Fmoc–Glu(O*t*Bu)-OH, Fmoc–Asn(Tr*t*)-OH, Fmoc–D-Thr(Bu*t*)-OH, and Fmoc–Tyr(Bu*t*)-OH. For support we have used Rink amide MBHA 1% DVB resin, with substitution typically ranging from 0.2–0.8 meq/g. Best results (e.g., highest purities of the crude glycopeptides) were obtained by coupling well below the resin capacity and then capping the excess capacity with acetic anyhydride (Ac_2O). Coupling of the Fmoc–amino acids was achieved using manual coupling methods with or without microwave heating or, typically, using mechanization, with extended reaction times or heating only for coupling of the glycosidic residue and the residue following, which may be regarded as an extremely hindered case of amino acid coupling. Use of NMP as a solvent rather than the more polar DMF also aided these couplings. Manual coupling reactions and critical couplings performed during mechanized coupling were typically monitored using Kaiser's ninhydrin test.

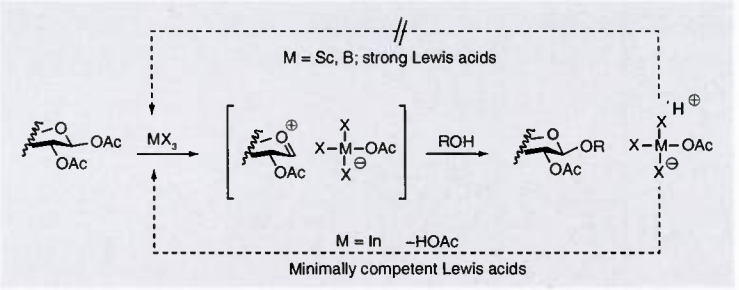


Figure 4. Minimally competent Lewis acids as glycosidation promotors. Lewis acids such as $InBr_3$ can dissociate (lower pathway) from the displaced acetate to form acetic acid and regenerate the Lewis acid catalyst. Stronger Lewis acids remain associated with the acetate (upper pathway) to produce a Brønsted acid and, generally, require a full equivalent of the Lewis acid.

The Fmoc group was removed from the *N*-terminus of the growing glycopeptide chain using a mixture of 3% piperidine and 2% diaza-1,3-bicyclo[5.4.0]-undecane in DMF for 10 min with argon bubbling as agitation. The acetyl protecting groups of the glycosides and the N-terminal Fmoc could be removed with 80% hydrazine hydrate ($H_2NNH_2 \cdot H_2O$) in CH_3OH with argon agitation 3X for approximately 2 h. A superior method used Boc protection for the last amino acid, which survived the hydrazine treatment but was removed by TFA. The synthetic glycopeptides were cleaved from the Rink resin with a 'TFA cocktail', F_3CCOOH:Et_3SiH:H_2O:$PhOCH_3$:CH_2Cl_2 (8:0.5:0.5:0.05:1), which also removed the side chain protection, and the N-terminal Boc group, if that method is employed. The crude glycopeptides were precipitated in cold diethylether, redissolved in H_2O and then lyophilized prior to reverse-phase HPLC. A preparative scale C_{18} column (Phenomenex® 250 × 22 mm, 250 × 55 mm or equivalent) was used, with an acetonitrile–water (CH_3CN-H_2O) gradient containing 0.1% TFA to obtain glycopeptides of greater than 97% purity. Homogeneity of the pure glycopeptides was confirmed by analytical reverse-phase HPLC and MS.

Glycopeptide analgesics based on enkephalins

Our initial attempts at improving the CNS bioavailability of opioid peptides came from collaborations between the Chemistry and Pharmacology Departments at the University of Arizona led by Victor Hruby [62]. In 1983

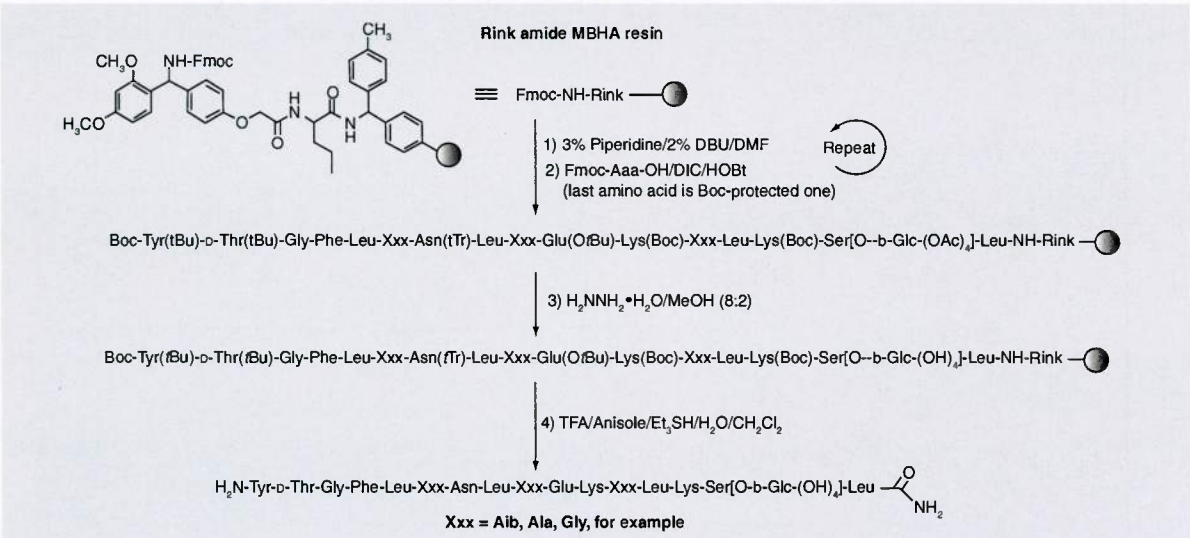


Figure 5. Glycopeptide assembly. MBHA-functionalized Rink polystyrene resin was used to provide the C-terminal amides upon cleavage after classical Fmoc construction of the glycopeptides. Treatment with hydrazine hydrate ($H_2NNH_2•H_2O$) in methanol (CH_3OH) was required to remove the acetates from the glycoside moiety prior to cleavage from the Rink resin. A Boc-protected amino acid may be used for the final amino acid (O-tBu-Tyr), which is cleaved with the TFA cocktail.

this group successfully synthesized and characterized a series of cyclic penicillamine containing enkephalin analogues (e.g., DPDPE) that had higher affinity and selectivity for DOP [63]. Incorporation of an unnatural amino acid (D-penicillamine) and the cyclic constraint into the peptide enhanced both its stability and DOP selectivity. One hypothesis was that by increasing the lipophilicity of an already quite lipophilic DPDPE, BBB penetration could be increased, which was confirmed in an *in vitro* BBB model that used bovine brain microvessel endothelial cells [64].

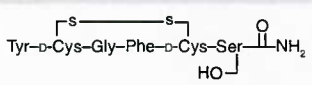
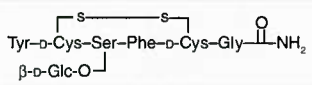
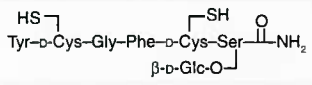
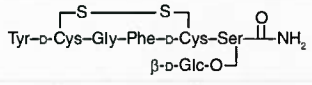
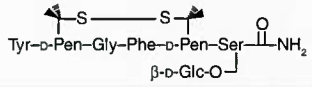
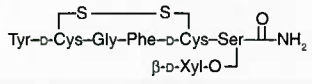
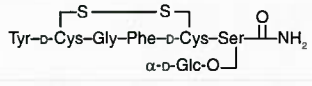
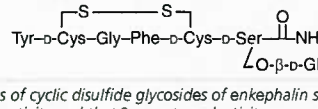
Concurrently, we tested an alternative hypothesis, which, in retrospect, was naive and incorrect, whereby attachment of a glucose molecule to the modified enkephalin peptide would make the overall ligand a substrate for the Glut-1 transporter [65]. In an effort to enhance CNS bioavailability, we synthesized a series of enkephalin analogues (**Tables 2–4**). It was predicted that CNS delivery of the enkephalin molecule across the BBB would be increased, and that we would observe antinociceptive activity following systemic administration. While the Glut-1 hypothesis eventually proved to be incorrect, the enkephalin glycosides did penetrate the BBB very effectively and produced potent and long-lasting antinociception in mice after intravenous (iv.) or intraperitoneal (ip.) injection [66].

Table 2 highlights some of the initial enkephalin glycopeptides that were synthesized [67]. Glycoside placement proved critical for affinity as determined by radioligand binding studies, and efficacy, as determined by GPI and MVD assays. Glycosylation sites close to the N-terminus resulted in reduced affinity for both DOP and MOP. Extension of the modified enkephalin peptide at the C-terminus allowed for glycosylation while preserving opioid receptor affinity, with some compounds retaining moderate DOP selectivity while others had approximately equal affinity for DOP and MOP.

Two glycopeptides, β-glucoside **3** and **4** (**Table 2**) were tested in mice for their ability to produce CNS-mediated antinociception after systemic administration, and were compared with the unglycosylated peptide control **1**. Both glycopeptides **3** and **4** produced dose- and time-related antinociception following ip. injection into mice, whereas the unglycosylated control peptides did not produce any measurable effects. The primary obstacles for better characterization of these glycopeptides were the somewhat tedious synthesis of the cyclic disulfides and the relatively low potency of the compounds (~30 mg/kg A_{50} values).

Larger quantities of a linear enkephalin glycoside based on Roques's so-called 'delta-enkephalin' or DTLET (YtGFLT,

Table 2. Cyclic disulfides related to DPDPE[1].

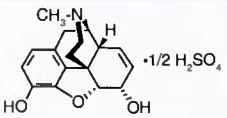
Compound	Structure	IC_{50} (nM)			
		δ receptor	μ receptor	MVD	GPI
1		6.1–6.4	30–43	5.5	26
2		3900	7700	520	3700
3		9.9	42	24	110
4		26–46	45–53	13	60
5		85	48,000	560	40,000
6		32	19		
7		10	53.3		
8		48	9		

[1]*Study of a series of cyclic disulfide glycosides of enkephalin showed that placement of the glycoside was critical to maintain opioid activity and that δ receptor selectivity was reduced in this series of compounds.*
GPI: Guinea pig ileum; MVD: Mouse vas deferens.

Tyr-D-Thr-Gly-Phe-Leu-Thr) were produced. The parent unglycosylated peptides, **9** and **10**, retained high affinity for both DOP and MOP, relatively weak binding to KOP, and displayed a slight preference (~tenfold) for functional activity in the mouse MVD assay versus GPI assay [68]. Both compounds were extremely potent (<0.1 nmol A_{50} values) following intracerebroventricular (i.c.v.) administration but required very large doses iv. to produce any antinociception in the mouse 55°C tail-flick. The addition of a glucoside to a serine in the sixth position of the peptide resulted in retention of modest selectivity for DOP over MOP in functional MVD/GPI tissue assays and in receptor-binding studies [69]. Both the parent peptide **(9)** and glycosylated analogue **(12)** were extremely potent in the mouse tail-flick assay following intracerebroventricular injection. However, glycopeptide **12** was significantly more potent following systemic routes of administration (iv., ip. and sc.). *In situ* BBB studies in rats also indicated, despite the increase in MW and increased water solubility, that the glycopeptide penetrated the BBB more effectively than its unglycosylated peptide counterpart **9** [70]. When compared with morphine, glycopeptide **12** resulted in lower levels of physical dependence as indicated by naloxone-precipitated withdrawal.

In an effort to further explore the structure-activity of glycosylation [71], a number of glycopeptides were synthesized to determine if the type of monosaccharide altered the transport characteristics and systemic potency of the lead peptide pharmacophore; if di- or tri-saccharides provided any additional benefit to pharmacokinetic and pharmacodynamic properties; and if bis- or tris-monosaccharides were viable alternative strategies for improving BBB transport and systemic potency [72]. In addition, several other modifications were made to explore the geometry of the attachment point (D vs L amino acid) of the glycoside and to see if the more sterically hindered threonine attachment differed from serine in its effects on activity. It should be noted that we stayed with the linear enkephalin

Table 3. Linear glycopeptide amides as δ receptor/μ receptor opioid agonists.

Compound	Structure	K_i (nM)			A_{50}	
		δ receptor	μ receptor	κ receptor	Intracerebroventricular (nmol)	Intravenous (μmol/kg)
Morphine		290 ± 38	0.79 ± 0.12	12.0 ± 1.3	2384	7.84
9	Tyr–D-Thr–Gly–Phe–Leu–Ser(HO)–NH_2	4.1 ± 41	1.4 ± 0.08	34.0 ± 2.2	0.068	46.4
10	Tyr–D-Thr–Gly–Phe–Leu–Thr(HO)–NH_2	9.71	11.7	ND	0.038	32.6
11	Tyr–D-Thr–Gly–Phe–Leu–Ser(β-D-Xyl-O)–NH_2	46.0	65.8	ND	0.092	9.45
12	Tyr–D-Thr–Gly–Phe–Leu–Ser(β-D-Glc-O)–NH_2	7.0 ± 1.2	2.4 ± 0.017	49 ± 4.3	0.023	11.4
13	Tyr–D-Thr–Gly–Phe–Leu–Ser(α-D-Man-O)–NH_2	23.0	15.2	ND	0.033	16.7
14	Tyr–D-Thr–Gly–Phe–Leu–Thr(β-D-Glc-O)–NH_2	16.8	39.8	ND	0.022	ND
15	Tyr–D-Thr–Gly–Phe–Leu–D-Ser(O-β-D-Glc)–NH_2	54.4	297.8	ND	0.035	ND
16	Tyr–D-Thr–Gly–Phe–Leu–D-Thr(O-β-D-Glc)–NH_2	24.5	31.8	ND	0.040	22.0

Glycopeptide monosaccharides based on DOP-preferring enkephalin sequences developed by BP Roques, DtGFLS (DTLES) and DsGFLT (DSLET).
ND: Not determined.

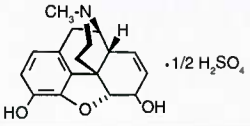
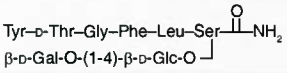
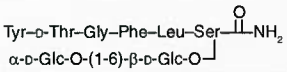
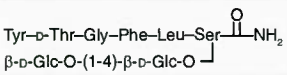
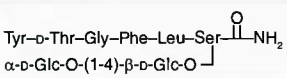
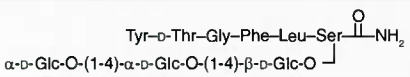
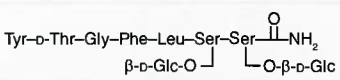
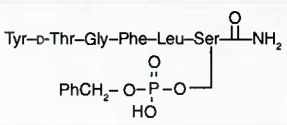
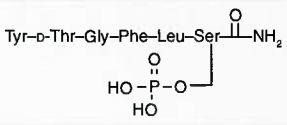
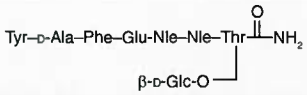
parent peptide as it had roughly equal affinity for DOP and MOP receptors. This was important in assessing potential effects of glycosylation on preferential biasing for DOP or MOP.

The initial Roques-based linear peptides tested had either an L-Ser or L-Thr added to the sixth position of the peptide. The geometry of the glucoside attachment did not impact functional potency/efficacy in the *in vitro* or *in vivo* assays (L-Ser vs D-Ser or L-Thr vs D-Thr). For the monosaccharides, the β-xylose was approximately two-times more potent than β-glucose or α-mannose following iv. administration. The three disaccharides (β-lactose, β-maltose and β-melibiose) were all more potent than the best monosaccharide tested, with the β-melibioside being the most potent of the three.

Based on these results, we synthesized additional glycopeptides that incorporated a trisaccharide (β-maltotriose) to see if additional size/bulk of the carbohydrate moiety would lead to further increases in iv. potency. The experimental data indicated a modest fall off in binding affinity and potency in the *in vitro* and *in vivo* functional assays. We also extended the hexapeptide to include one to two additional Ser or Thr attachment points with β-glucose (bis- and tris-monosaccharides) to more fully explore the structure–activity relationship. In all cases, the additional glycosyl bulk reduced potency following i.c.v. administration, and the one compound tested iv. was significantly less potent than the original glycopeptide (β-glucoside and L-serine attachment).

Additional studies confirmed aspects of the *in vivo* studies [67]. Larger carbohydrates reduced octanol:saline partitioning (logD value), indicating greater water solubility (parent peptide < monosaccharide < disaccharide < triscchariide). Serum and brain stability of the glycopeptides

Table 4. Linear glycopeptide amides as μ receptor/δ receptor opioid agonists.

Compound	Structure	K_i (nM)			A_{50}	
		δ receptor	μ receptor	κ receptor	Intracerebro-ventricular (nmol)	Intravenous (μmol/kg)
Morphine		290 ± 38	0.79 ± 0.12	12.0 ± 1.3	2384	7.84
17		9.20 ± 1.70	5.00 ± 0.65	42.0 ± 5.0	0.018	3.20
18		6.48	41.9	ND	0.034	2.16
19		13.0 ± 0.55	4.5 ± 0.12	31.0 ± 0.3	ND	ND
20		9.86	30.8	ND	0.062	6.82
21		25.0	56.7	ND	0.061	10.9
22		ND	ND	ND	0.380	140.8
23		3.3 ± 0.33	8.7 ± 0.93	130 ± 6.2	0.093	30% at 32.0 mg/kg
24		7.9 ± 0.73	3.9 ± 0.19	310 ± 66	0.34	54.1
25		14.0 ± 0.34	1100 ± 13	3% at 10.0 μM	0.22	5.63

Glycopeptide disaccharides, and phosphates based on δ receptor-preferring enkephalin sequences developed by BP Roques, DtGFLS (DTLES) and DsGFLT (DSLET). ND: Not determined.

also increased with these substitutions. In an *in situ* model of BBB transport the disaccharide proved to be the most readily transported with the trisaccharide having a reduced R_{Br} value (though still superior to the unglycosylated control). We extended the *in vivo* work by adding additional pain assays to assess efficacy. The disaccharide **17** produced potent antinociception in the formic acid, acetic acid and carrageenan assays following systemic administration (all of these pain assays have an inflammatory component).

Based on this modest library of glycopeptides, we chose the β-lactoside **(17)** as the lead molecule to pursue more advanced *in vivo* characterization. While not the most potent of the disaccharides, the compound was much easier and less costly to synthesize compared with melibiose. Glycopeptide **17** also had some additional desirable characteristics, including being highly water soluble (>50 mg/ml). Based on these findings, we advanced **17** into a more complete characterization of its antinociceptive efficacy and side-effect profile.

As expected, **17** produced full efficacy in the GTPγS assay with a modest selectivity for DOP over MOP. This profile was similar to what was

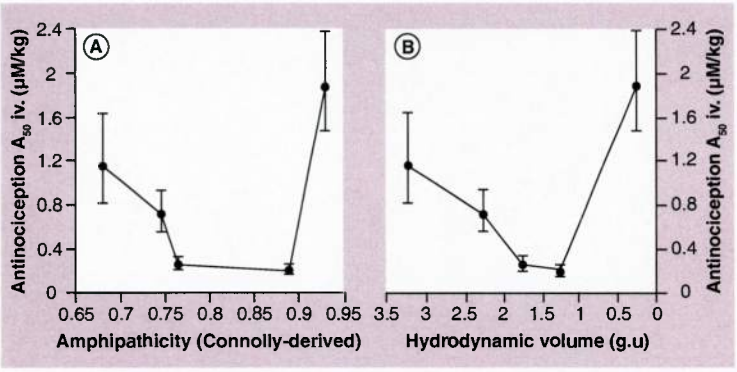


Figure 6. Antinociception studies indicate a U-shaped or V-shaped curve when the A_{50} potency values are correlated with predicted amphipathicity. The hydrodynamic values (glucose units) or Connolly-derived amphipathicity values are plotted along the X-axes, and A_{50} values derived from mouse intravenous tail-flick data are plotted on the Y-axis. Both analyses produce a U-shape or V-shape, as predicted by the biousian hypothesis [84]. The amphipathicity values were calculated using the formula $A = e^{-Awater/Alipid}$, where ^{Awater} = the Connolly surface area of the hydrophilic moiety (Å^2) and ^{Alipid} = the Connolly surface area of the rest of the lipophilic peptide message segment YaG(N-MeF).

observed in the functional MVD and GPI tissue assays. We confirmed this profile *in vivo* by pretreating mice with various opioid antagonists. The general antagonist naloxone completely blocked the actions of **17** in the 55°C tail-flick assay in mice. In contrast, the peripherally selective antagonist naloxone methiodide did not alter the agonist actions of the compound. Subtype selective DOP (naltrindole) and MOP (β-FNA) antagonists each partially blocked the antinociceptive actions of **17** and, when combined, they completely eliminated the agonist actions. The KOP-selective antagonist nor-BNI was without effect.

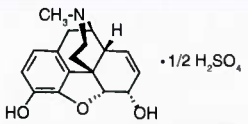
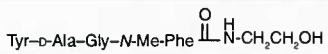
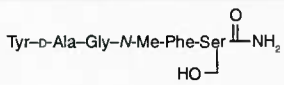
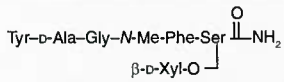
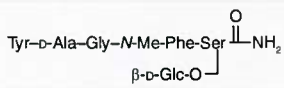
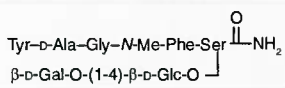
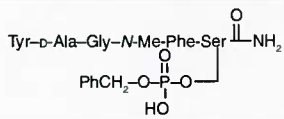
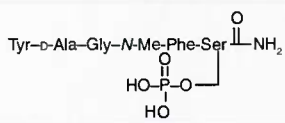
As a lead molecule, **17** was also tested in several rat models of pain to determine how broad an antinociceptive spectrum the compound might have. The first assay used was a post-surgical incision model of the hind paw [73]. Morphine and **17** both produced dose-related reversal of the tactile allodynia associated with the injury [D Giuvelis *et al.*, Unpublished Data]. On a µmol/kg basis, **17** was almost equal to morphine in terms of potency. Similar results were seen in a subchronic inflammatory pain model induced by complete Freund's adjuvant, although, in this case, **17** had greater potency than morphine, possibly due to enhanced DOP signaling under inflammatory conditions. Finally, **17** was compared with gabapentin in a rat spinal nerve ligation model of neuropathic pain. Glycopeptide **17** produced potent reversal of the tactile allodynia and thermal hyperalgesia post-ligation, whereas gabapentin only reversed tactile allodynia at the doses examined [D Giuvelis *et al.*, Unpublished Data]. Collectively, the data indicate that a mixed DOP/MOP agonist has a broad spectrum of antinociceptive effects in acute and chronic pain models, including ones that have inflammatory and/or neuropathic pain components.

One of the initial screens for side effect was to inject increasing doses of **17** or morphine and collect locomotor data in an automated open field assay. Morphine and other MOP agonists stimulate forward locomotion in imprinting control region mice. This effect becomes pronounced at near maximal and supramaximal antinociceptive doses. The mixed MOP/DOP agonist **17** produced an initial and transient decrease in forward locomotion that was replaced by a very mild stimulation of activity at later time points. We further investigated the initial inhibition of locomotor activity by pretreating mice with naloxone methiodide or nor-BNI. Both pretreatments attenuated the effects of **17** on locomotion and completely eliminated both effects when the two opioid antagonists were administered simultaneously. This indicated that stimulation of peripheral opioid receptors can produce a transient decrease in exploratory locomotor behavior and there may be a modest κ-agonist effect of the compound in the CNS that contributes to reduced stimulation of locomotor activity but does not contribute to the antinociceptive effects in the 55°C tail-flick assay.

Interestingly, one of the other gross observable differences between **17** and morphine is a lack of Straub tail and muscular rigidity with **17**. We quantified this effect in dose–response curves versus antinociception with both morphine and the mixed agonist **17**. The potential of morphine to produce both effects overlapped, whereas it took much higher doses of **17** to produce the muscular rigidity and Straub tail compared with its antinociceptive effects.

Based on the mixed DOP/MOP profile of **17**, we were interested in evaluating the tolerance and physical dependence liability of the compound relative to morphine. For tolerance studies, we used a common paradigm involving twice-daily injections of the approximate A_{90} doses of the agonist (or vehicle) for 3 days. On the morning of day 4, full dose–response curves were constructed for each compound in the agonist- and vehicle-treated animals. Repeated doses of morphine resulted in an approximately

Table 5. Glycopeptides based on DAMGO.

Compound	Structure	K_i (nM)			A_{50}	
		δ receptor	μ receptor	κ receptor	Intracerebroventricular (nmol)	Intravenous (μmol/kg)
Morphine		290 ± 38	0.79 ± 0.12	12.0 ± 1.3	2384	7.84
DAMGO		990 ± 35	0.56 ± 0.006	270 ± 9.3	30	1.88
26		600 ± 44	0.68 ± 0.02	190 ± 9.3	2.0	0.20
27		730 ± 66	1.30 ± 0.16	160 ± 10	2.0	0.27
28		54% at 10 μM	1.30 ± 0.14	270 ± 2.5	19	0.72
29		1600 ± 129	0.66 ± 0.05	350 ± 51	2.0	1.15
30		55 ± 4.6	4.2 ± 0.34	570 ± 9.8	33	30% at 38.1 mg/kg
31		1400 ± 180	3.8 ± 0.73	31% at 10 μM	42	1.5

Glycopeptide disaccharides and phosphates based on the μ receptor-preferring sequences.

13-fold rightward shift in the A_{50} value indicating substantial development of antinociceptive tolerance. Equivalent doses of **17** (in terms of analgesia) resulted in a significantly reduced rightward shift (<fivefold). Morphine and glycopeptide **17** have similar durations of action and AUC values, making the comparisons more straightforward.

For assessment of physical dependence liability, we used both an acute (single high-dose administration of agonist) and chronic (twice-daily injections for 3 days) dependence protocol. In both cases, injection of the general opioid antagonist naloxone was used to precipitate withdrawal and several indices of withdrawal were recorded (vertical jumps and paw tremors, for example). The level of physical dependence/severity of withdrawal was consistently lower with the **17** exposure compared with equivalent exposures of morphine [74]. The working hypothesis for explaining these results is that the antinociceptive effects of the mixed DOP/MOP compound synergize at the cellular or network level, whereas the processes that drive tolerance and/or physical dependence are additive or subadditive. A predominantly MOP-selective agonist, on the other hand, requires significant occupation of the MOP receptors at sites both responsible for antinociception and tolerance/dependence. Other explanations are possible, including the formation of heterodimers with the glycopeptide **(17)** versus the small molecule (morphine) that lead to activation of different signaling pathways.

To further characterize the side-effect profiles of the glycopeptide, two commonly used assays for assessing MOP effects were used (gastrointestinal transit and respiratory depression). We

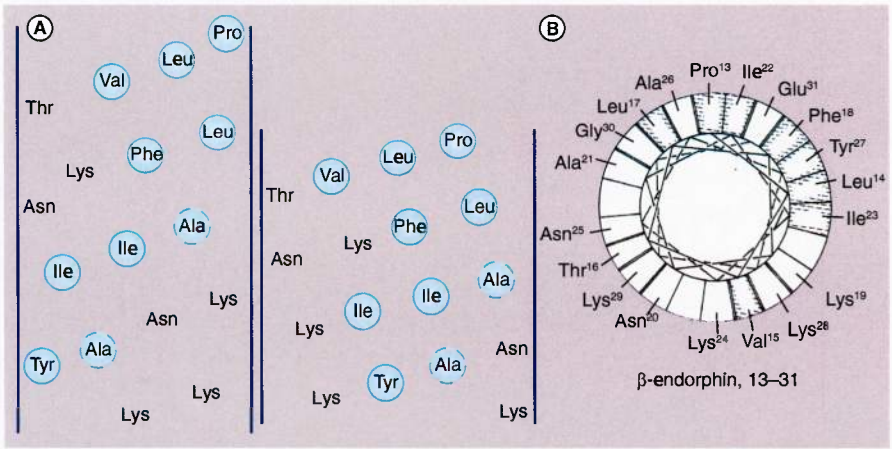


Figure 7. Representations of the amphipathic helical region in β-endorphin.
(A) Human β-endorphin [12–28] represented both as an α-helical net projection (left) and as a π-helical net projection (right) [90]. The lipophilic (hydrophobic) residues are circled. **(B)** Human β-endorphin [12–30] represented as an axial projection of a π-helix [91].

had predicted that the DOP/MOP profile would have a reduced effect on these parameters compared with equivalent doses of morphine. This was not the case. Glycopeptide **17** also inhibited upper-gastrointestinal transit and suppressed respiratory response to elevations in CO_2 on the minute ventilation parameter. The former may have been due to the apparent higher concentrations of glycopeptide **17** in the peripheral circulation compared with CNS, thus, overwhelming the MOP populations in the enteric nervous system. These values were estimated from the i.c.v. versus iv. potency ratios to produce antinociception for **17** versus morphine (more formal pharmacokinetic measures are currently being conducted). The respiratory depression observations indicate that a slight preference for DOP over MOP is not sufficient to differentiate from a MOP selective agonist.

Additional studies were conducted with **17** with respect to its abuse liability. As mentioned previously, the level of locomotor stimulation with **17** was markedly reduced compared with morphine. The stimulation of forward locomotion is generally interpreted as an activation of mesolimbic dopamine systems and an indicator of abuse liability. Our group has also conducted preliminary studies using conditioned place preference and iv. drug self administration in rodents. In the conditioned place preference studies, morphine produced a significant place preference whereas antinociceptive equivalent

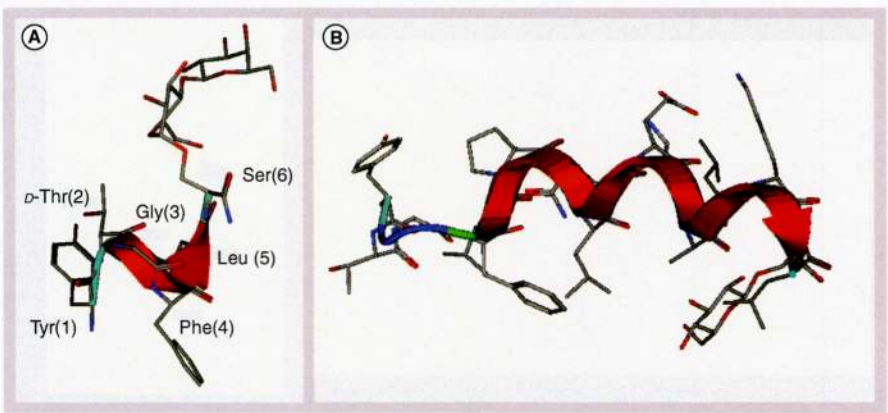


Figure 8. Micelle-bound structures of glycopeptide analogues determined by NMR related to (A) enkephalins and (B) endorphins [92,93].

Table 6. The amphipathic helix address†.

Compound	Message sequence	Link	Helical address sequence	K_i (nM)			A_{50}	
				δ receptor	μ receptor	κ receptor	Intracerebro-ventricular (nmol)	Intravenous (μmol/kg)
32	YtFGL	P	NLBEKALKSL-NH_2	9.6	2.3	2.8	0.57	0.32
33	YtFGL	P	NLBEKALKS*L-NH_2	3.6	2.6	2.3	0.58	0.36
34	YtFGL	P	NLBEKALKS**L-NH_2	12.0	8.2	5.7	0.11	1.06
35	YtFGL	GABA	SL-NH_2	16	11	38	0.064	(>> 10)
36	YtFGL	GABA	S*L-NH_2	39	16	56	0.059	2.29
37	YtFGL	GABA	NLBEKALKSL-NH_2	29	31	49	0.143	1.12
38	YtFGL	GABA	NLBEKALKS*L-NH_2	4.2	4.3	11	0.046	0.28
39	YtFGL	GABA	NLBEKALKS**L-NH_2	4.3	0.97	11	0.165	1.13
40	YtFGL	DAVA	NLBEKALKSL-NH_2	32	37	64	0.162	1.41

*†For endorphin analogues **32**, **33** and **34**, the glycosylation state showed only minor effects on binding and intravenous potency. The addition of a carbohydrate (**35** vs **36**) clearly shows a dramatic effect on intravenous potency, presumably by affecting transport, and combining the two features into the address region (e.g., **38**) provides the best delivery.*
S = OH; S = Glc; S** = Lact.*
DAVA: Delta amino valeric acid; GABA: Gamma amino butyric acid; P: Proline.

doses of **17** did not (similar to vehicle). In rat self-administration studies **17** maintained significantly lower numbers of infusions than the more MOP selective agonists morphine and fentanyl. In addition, the cumulative latency to delivery of the first three infusions of **17** (at the peak of the dose–effect curve) was significantly longer than morphine and fentanyl [Stevenson *et al.* Manuscript in Preparation]. These experiments suggest that the reinforcing effects of **17** are less than the prototypical MOP agonists morphine and fentanyl. The reinforcing effects of **17** were also evaluated in rhesus monkeys [75]. Under the conditions examined **17** did not support self-administration in rhesus across a series of doses/concentrations, although the results are more difficult to interpret due to species differences with respect to pharmacokinetics.

Current studies with DOP-selective peptides

With respect to our analgesic drug-development efforts, the prior work with **17** (mixed DOP/MOP agonist) indicated to us that greater DOP selectivity might be needed in order to differentiate a lead candidate from currently available MOP analgesics. This was based not only on the extensive characterization we had done with **17**, but also the literature demonstrating involvement of DOP receptors in neuropathic and other chronic pain states, and the further improvement in side-effect profiles [76–78]. We have also been interested in exploiting potential differences between the small-molecule DOP agonists, such as BW373U86 and SNC80, and the larger peptide-based deltorphin II analogues [79–82]. The former are ineffective in acute nociceptive assays that have high stimulus intensities, whereas the later are effective. This may be due to differences in ligand/receptor biasing or interactions with unique homo- or hetero-dimers of the DOP [83].

A series of glycosylated deltorphin analogues were synthesized that retained their high affinity, selectivity and efficacy at the DOP. Through an *in vitro* screening process, two to three lead compounds emerged, including glycopeptide

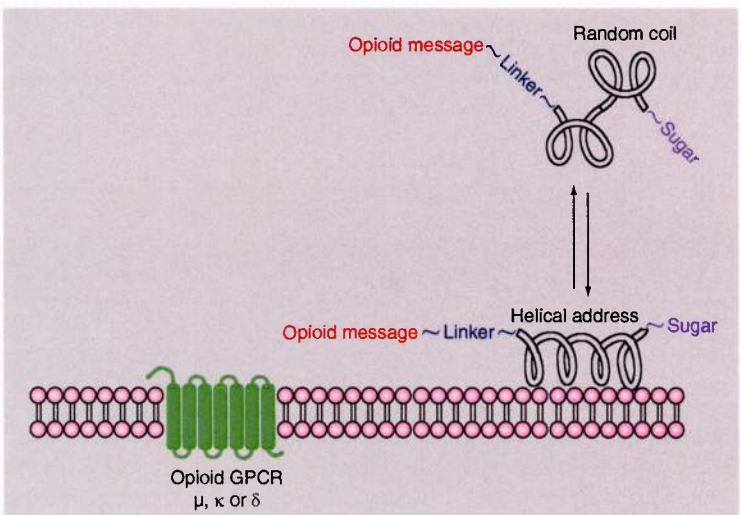


Figure 9. Biousian behavior in a helix context. Modulation of amphipathic helix stability should modulate interactions with biological membranes and 'searching' for the receptor.

25. (**TABLE 4**) This compound exhibits a low nM EC_{50} value in the GTPγS assay and is very potent when injected i.c.v. (<1 nmol A_{50} value) (KITSOS *ET AL.* MAUSCRIPT IN PREPARATION). Glycopeptide **25** is also systemically bioavailable following iv. and p.o. dosing, although the oral dose requires a proprietary co-formulation technology developed by Unigene. We are currently conducting further assessments of efficacy and side effects of glycopeptide **25** and comparing them to morphine and to **17**. These preliminary data suggest that we the efficacy of the δ-agonist **25** is at least equal to the mixed μ/δ-agonist **17** with a side-effect profile on gastrointestinal transit that is superior to morphine and **17**.

DAMGO-based MOP agonists

To further explore and exploit the biousian hypothesis [84], the classical μ-agonist (K_i: 0.53 nM) DAMGO [85,86] was used as a lipophilic peptide message [87], and additional moieties added to provide a water soluble address to produce a series of MOP-selective ligands. Their pharmacology was assessed *in vitro* and *in vivo*

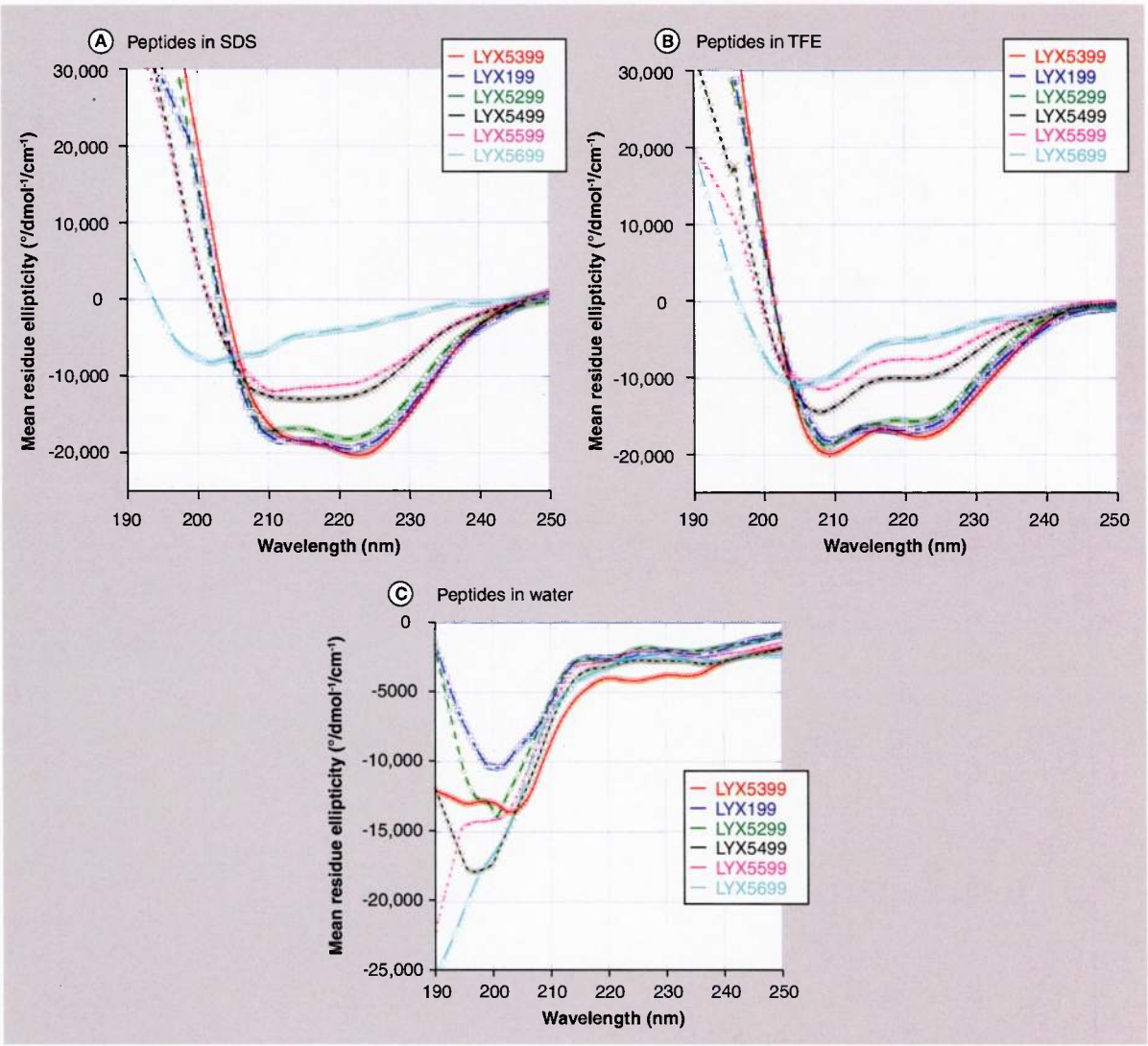


Figure 10. Circular dichroism to measure helicity. Modulation of the amphipathic helix stability can be directly measured by examining the circular dichroism behavior in **(A)** sodium dodecylsulfate, **(B)** trifluoroethanol and **(C)** water, and measuring the ellipticity at 222 nm.

[88,89]. It was believed that the exploration of the biousian hypothesis [84] within the context of a pure MOP agonist would simplify interpretation of the results (**FIGURE 6**). The binding and antinociceptive effects of **26–29** are shown in **TABLE 5** with values for morphine sulfate and DAMGO included for comparison. Binding was determined in Chinese hamster ovary cell membranes as before. Antinociception (A_{50} values) was determined after i.c.v. or iv. administration using the mouse 55°C tail-flick assay.

Since the binding affinities and receptor preferences of the μ-selective DAMGO derivatives are similar, the analgesic potencies of the glycopeptides are largely determined by their ability to penetrate the BBB by transcytosis [67], which in turn depends on the biousian character of the drugs [84]. One may consider two extremes that result in poor delivery of a peptide drug:.

- The peptide binds tightly to biological membrane, effectively removing it from solution;
- The peptide remains in aqueous solution, effectively preventing it from binding to biological membranes.

Thus, the goal in producing glycopeptides that are capable of effective CNS delivery, binding and GPCR activation, is to balance the degree of glycosylation, which effectively determines the amount of time the glycopeptide spends on the endothelial membrane of the BBB, as well as other membranes that the glycopeptide is likely to encounter. Affinity for the membrane is still required for effective binding and activation of the GPCR but a certain amount of membrane hopping is required for effective drug transport. Thus, a plot of the BBB transport or antinociceptive A_{50} values versus the membrane affinity produced a U-shaped or V-shaped curve (**FIGURE 6**).

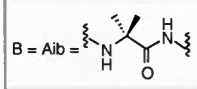
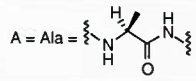
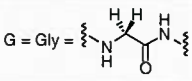
Unpublished studies in mice showed that disaccharide **29** produced behaviors (Straub tail and hyperlocomotion) suggestive of 'narcotic intoxication' at very low doses and had an extreme addiction liability as indicated by naloxone precipitated withdrawal studies. While μ-agonists, such as peptide **26** or glycopeptide **27**, could provide some useful clinical features that that morphine and other μ-selective analgesics do not possess, there does not seem to be much appetite for adding drugs to the pharmacopeia with side-effect profiles worse than morphine, and that are likely to be extremely addictive.

Kaiser's pioneering studies on the structure of β-endorphin

The late Emil Kaiser led a group of researchers at the Rockefeller Institute (USA) in studies of the structure and function of naturally occurring peptides, focusing their pioneering efforts on endogenous opioid hormones of mammalian origin including β-endorphin [90] and other seemingly diverse peptides from arthropods such as bee venom, or melittin (**FIGURE 7**) [91]. In fact, both β-endorphin and melittin interact strongly with biological membranes. It is in understanding the similarities and differences in exactly how these two peptides interact with

Table 7. Intrinsic helix stability in H_2O[†].

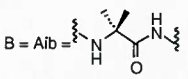
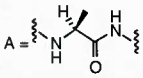
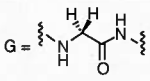
Series	Message sequence	Link	Helical address sequence	Helicity per residue in H_2O buffer (%)		
				S° = OH	S* = Glc	S** = Lact
41	YtFGL	P	NLBEKBLKS°/*/**L-NH_2	*Not soluble*	4.5	0.7
42	YtFGL	P	NLBEKALKS°/*/**L-NH_2	12.2	7.5	1.0
43	YtFGL	P	NLAEKBLKS°/*/**L-NH_2	7.4	7.0	1.3
44	YtFGL	P	NLAEKALKS°/*/**L-NH_2	7.2	6.9	0.1
45	YtFGL	P	NLAEKGLKS°/*/**L-NH_2	8.7	0.0	0.0
46	YtFGL	P	NLGEKALKS°/*/**L-NH_2	7.1	0.8	0.0
47	YtFGL	P	NLGEKGLKS°/*/**L-NH_2	8.1	0.7	0.0

B = Aib =  A = Ala =  G = Gly = 

[†]Variation in the intrinsic helicity was achieved with minimal impact on other properties by altering only two amino acid residues with B (Aib), A (Ala) or G (Gly). Water solubility could be affected by altering the glycosylation state of the S. Except for the extremely helical peptides without glycosylation, all of these compounds were highly water soluble, and showed only random coil behavior in aqueous solution.
Aib: α-aminoidobutyric acid; P: Proline; S: Serine.

Table 8. Amphipathic helix stability in the presence of micelles[†].

Compound	Message sequence	Link	Helical address sequence	Helicity per residue in sodium dodecyl sulfate micelles (%)		
				S° = OH	S* = Glc	S** = Lact
41	YtFGL	P	NLBEKBLKS°/*/**L-NH_2	70	56.7	40.8
42	YtFGL	P	NLBEKALKS°/*/**L-NH_2	60.8	44.9	35.7
43	YtFGL	P	NLAEKBLKS°/*/**L-NH_2	58.5	35.7	33.6
44	YtFGL	P	NLAEKALKS°/*/**L-NH_2	54.4	35.7	31.8
45	YtFGL	P	NLAEKGLKS°/*/**L-NH_2	37.8	24.6	13.3
46	YtFGL	P	NLGEKALKS°/*/**L-NH_2	33.0	14.5	4.5
47	YtFGL	P	NLGEKGLKS°/*/**L-NH_2	11.0	6.4	2.8

B = Aib =  A =  G = 

[†]*Helix amphipath stability was greatly altered in the presence of sodium dodecyl sulfate by altering the two amino acid residues with B (Aib), A (Ala) or G (Gly). Water solubility and the degree of helix formation (%-helix/residue) was also affected by altering the glycosylation state of the S.*
Aib: α-aminoidobutyric acid; P: Proline; S: Serine.

membranes that will provide a rationale and opportunity for improving peptide drug delivery and effective design of CNS drugs. Our group has incorporated a design strategy for BBB penetration that exploits amphipathic α-helices that can interact strongly with cellular membranes to enhance endocytotic events. Critically, this biousian approach also preserves a degree of hydrophilicity for the overall peptide, especially when it is not interacting with the phospholipid membrane [84].

All of our glycosylated enkephalins display well-defined secondary structures in sodium dodecyl sulfate (SDS) micelles, irrespective of the identity of the glycoside attached to the peptide, which represent the simplest model for a biological membrane. In contrast, no defined structure is observed in aqueous media (**Figure 8A**) [92]. Similarly, the longer glycopeptides related to β-endorphin tend to adopt amphipathic helical structures in SDS micelles as well as phospholipid bicelles (**Figure 8B**) [93]. These larger glycopeptides displayed an ensemble of random coil conformations in aqueous solvent despite they are 17 residues in length. It is evident from these observations that amphipathicity of the glycopeptide (not simply hydrophilic or hydrophobic nature alone) is essential for membrane transport. This biousian behavior (i.e., random coil state in aqueous environment and highly folded state in membrane environment) appears essential for the effective transport of these larger peptides across the BBB [84].

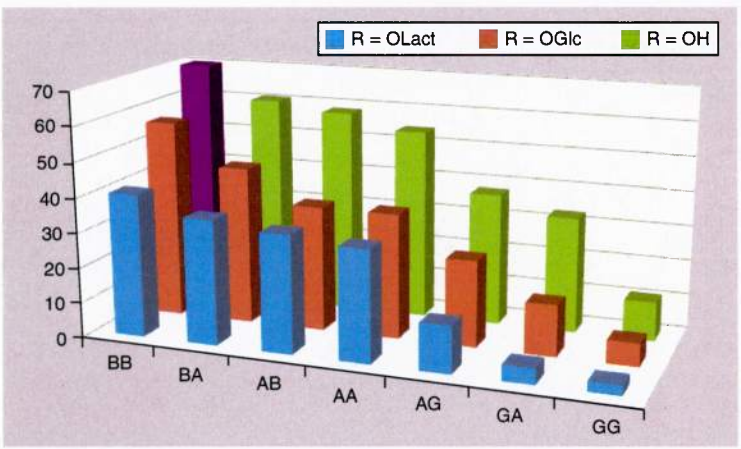


Figure 11. Helicity in sodium dodecyl sulfate as a function of peptide address sequence and glycosylation state.

Biousian behavior has also been observed for other endogenous GPCR peptide ligands [84,94]. Many, if not all peptide ligands that activate GPCRs lack a well-defined structure in aqueous buffer, but tend to fold into largely α-helical conformations in the presence of organic solvents [95] and lipid micelles [96], and in crystals [97,98]. Inooka and coworkers have been able to demonstrate that micelle-bound conformation of a peptide ligand is closely related to the actual receptor-bound conformation, that is, the α-helical region is similar in both cases [99].

Glycopeptide opioids based on endorphins

Initially, we focused on the importance of the glycoside in the context of the short enkephalin-based glycopeptides to enhance membrane hopping and BBB penetration, as evidenced by

Table 9. Binding and intracerebroventricular antinociception as a function of helix stability – unglycosylated peptides.

Compound	Message sequence	Link	Helical address sequence	K_i (nM)			A_{50}
				δ receptor	μ receptor	κ receptor	Intracerebroventricular (nmol)
41*	YtFGL	P	NLBEKBLKSL-NH_2	16	8.7	27	1.66
42*/32	YtFGL	P	NLBEKALKSL-NH_2	9.6	2.3	2.8	0.57
43*	YtFGL	P	NLAEKBLKSL-NH_2	9.7	10	28	0.18
44*	YtFGL	P	NLAEKALKSL-NH_2	3.5	2.8	5.5	0.55
45*	YtFGL	P	NLAEKGLKSL-NH_2	15	17	49	0.79
46*	YtFGL	P	NLGEKALKSL-NH_2	14	21	23	0.96
47*	YtFGL	P	NLGEKGLKSL-NH_2	19	14	17	1.92

S = OH; S* = Glc; S** = Lact.
P: Proline; S: Serine.

peptide **35** compared with glycopeptide **(Table 6)** [35]. The much larger endorphin-based peptides did not always require glycosylation in ordet to achieve effective membrane hopping rates to produce effective drug transport **(Figure 9)**. In fact, the glycosylation could have no effect (**32** vs **33**), or even a deleterious effect on iv. potency (**32** vs **34**). We think that the flexibility of the linker (proline vs GABA vs DAVA) may play an important role as well, satisfying both the GPCR and the membrane binding requirements. Mote impottantly although, the intrinsic stability of the membrane bound helix can decisively affect drug transport, as evidenced by iv. potency *in vivo*.

To determine the effect of helix stability a series of opioid C-terminal amide peptide 16-mers **41–47** were prepared using seven different helical segments with a minimal pertutbation of the structure. Two amino acid residues (position 8 or 12) wete substituted with one of three amino acids; helix-weakening glycine (Gly or G); helix-preferring L-alanine (Ala or A); ot strongly helix-preferring α-aminoisobutyric acid (Aib or B) to produce seven different peptides of decreasing helix stability (B~B, B~A, A~B, A~A, A~G, G~A and G~G). Each series of helices was prepared in three different glycosylation states; one peptide series with unglycosylated L-serine (S°); a series of monosaccharides bearing a single β-*O*-D-glucose (Glc) on L-serine (S*); and a series of disaccharide glycopeptides bearing β-*O*-lactose (Lact) on L-serine (S**). These closely related seven peptides and 14 glycopeptides were characterized by high field (600 MHz) NMR in the presence of SDS and D_2O/H_2O, and by circular dichroism in H_2O, H_2O/F_3CCH_2OH (data not shown here), and SDS/H_2O **(Figure 10)** [100,101]. The degree of helicity was conveniently expressed as%-helicity per residue [102,103]. Note that helicity was essentially absent in aqueous media **(Table 7)**. Peptide **41** was not soluble in H_2O. However, in the presence of SDS micelles there was a clear trend,

Table 10. Binding and intracerebroventricular antinociception as a function of helix stability – glucopeptides.

Compound	Message sequence	Link	Helical address sequence	K_i (nM)			A_{50}
				δ receptor	μ receptor	κ receptor	Intracerebroventricular (nmol)
41*	YtFGL	P	NLBEKBLKS*L-NH_2	11	9.1	6.9	1.13
42*/33	YtFGL	P	NLBEKALKS*L-NH_2	3.6	2.3	2.6	0.58
43*	YtFGL	P	NLAEKBLKS*L-NH_2	14	6.1	12	1.46
44*	YtFGL	P	NLAEKALKS*L-NH_2	7.9	6.2	8.9	1.44
45*	YtFGL	P	NLAEKGLKS*L-NH_2	6.3	2.2	6.3	1.47
46*	YtFGL	P	NLGEKALKS*L-NH_2	5.7	2.9	11	2.54
47*	YtFGL	P	NLGEKGLKS*L-NH_2	7.1	3.5	4.4	2.12

S = OH; S* = Glc; S** = Lact.
P: Proline; S: Serine.

Table 11. Binding, intracerebroventricular and intravenous antinociception as a function of helix stability – lactosides

Compound	Message sequence	Link	Helical address sequence	K_i (nM)			A_{50}	A_{50}
				δ receptor	μ receptor	κ receptor	Intracerebro-ventricular (nmol)	Intravenous (μmol/kg)
41**	YtFGL	P	NLBEKBLKS**L-NH_2	5.9	1.2	2.5	0.57	4.3
42/34**	YtFGL	P	NLBEKALKS**L-NH_2	12	8.2	5.7	0.11	1.1
43**	YtFGL	P	NLAEKBLKS**L-NH_2	14	3.2	3.5	0.21	1.2
44**	YtFGL	P	NLAEKALKS**L-NH_2	2.6	< 1	3.5	0.76	4.1
45**	YtFGL	P	NLAEKGLKS**L-NH_2	4.7	0.97	4.3	0.14	8.7
46**	YtFGL	P	NLGEKALKS**L-NH_2	13	4.4	6.6	0.50	
47**	YtFGL	P	NLGEKGLKS**L-NH_2	4.5	1.4	2.6	0.23	> 40

S = OH; S = Glc; S** = Lact.*
P: Proline; S: Serine.

(Table 8) with B–B strongly favoring helical conformations, G–G disfavoring helical conformations, and intermediate levels of helicity for the others in relatively smooth, although not monotonic, stepwise fashion (Figure 11). The most helical compound, **41**, was not soluble in water, but readily dissolved in the presence of SDS.

All 21 compounds were characterized in terms of δ/μ/κ-binding, and subjected to the 55°C tail-flick test in mice, both after i.c.v. administration (Tables 9–11). Administration with iv. bolus injection is currently being studied, and the data (not shown) is only preliminary. Although there were only modest variations in the binding selectivity, and in the A_{50} values obtained after i.c.v. administration (bypassing the BBB), there are large variations in the A_{50} values obtained after iv. injection, ranging from little or no antinociceptive activity observed at 32 mg/kg down to extremely potent activities with A_{50} values below 500 μg/kg.

Executive summary

- The three opiate receptors, μ, δ and κ receptors, and their endogenous peptide neurotransmitters, the enkephalins and endorphins, play numerous roles in the CNS. Principal among these roles is the modulation of acute and chronic pain states. The endogenous peptide neurotransmitters are highly amphipathic, spending the bulk of their independent existence after cleavage from larger precursor proteins and prior to release stored in vesicles contained within the presynaptic membrane. Upon release they float in the postsynaptic membrane where they bind and activate opiate receptors. The effects of the endogenous peptide ligands are local in nature due to their poor transport properties.
- Glycosylation of the relatively short enkephalins allows them to leave the membrane environment to engage in 'membrane hopping'. The key is to add a water soluble moiety to the peptide in such a way that in enhances water solubility without interfering with its interaction with the membrane. We have dubbed this effect 'biousian behavior' in which the glycopeptide may exist either in a relatively constrained membrane bound state, or an aqueous state as a 'random coil' ensemble. The degree of glycosylation can be adjusted to optimize the biological transport rates of the enkephalin-based glycopeptides to provide drugs based on the endogenous enkephalins.
- The biousian behavior permits the glycopeptides and related serine phosphates to penetrate the blood–brain barrier, probably via transcytosis.
- The larger endorphins have a message–linker–address peptide format. The message exists as a turn structure and is largely responsible for receptor binding and activation. The address exists as an amphipathic helix and is largely responsible for binding to the membrane. A flexible linker domain allows the receptor binding requirements and the membrane binding requirements to be met simultaneously. The address domain can be altered in order to optimize the biological transport rates of the endorphin-based glycopeptides based on the endogenous endorphins.
- Peptide sequences can be varied to produce glycopeptide drugs that favor any of the three opiate receptors, or various combinations, such as μ/κ receptors and mixed μ/δ-agonists. Side-effect profiles of the analgesics can be manipulated by altering the agonist features of the enkephalins or the endorphin message segments.
- Glycopeptide-based drugs have been developed that exert analgesic effects in mice far in excess of the classical narcotics, such as morphine, Demerol® and Oxycontin®. We are optimistic that interest in glycopeptide drugs derived from neuropeptides will increase, and that all major pharmaceutical companies will establish research programs in the area. We expect that the applications will not be limited to opiate agonists and that a large number of the 250 plus endogenous neuropeptides can be converted into glycopeptide drugs capable of penetrating the blood–brain barrier.

Future perspective

While it would be premature to state that we have a complete understanding of the role of amphipathicity, biousian behavior [84] and membrane affinity in the transport of opioid peptides and glycopeptides, it is clear that the transport of these compounds across biological membranes of interest is not only possible, but quite efficient. It seems clear that the observed transport phenomena will not be limited to this class of neuropeptides, as researchers are exploring glycosylated endomorphins [104], dermorphins [105], dermorphins [106] and other peptides [107] with good results. Thus, we remain optimistic that interest in glycopeptide drugs derived from neuropeptides will increase and that all major pharmaceutical companies will establish research programs in the area.

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